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PCT

WORLD INTELLECTUAL PROPERTY ORGANIZATION
International Bureau

INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 3 : C12N 15/00; C12P 21/02

A1

(11) International Publication Number: WO 84/ 00773

(43) International Publication Date: 1 March 1984 (01.03.84)

(21) International Application Number: PCT/SE83/00297

(22) International Filing Date: 23 August 1983 (23.08.83)

(31) Priority Application Number: 8204810-9

(32) Priority Date: 23 August 1982 (23.08.82)

(33) Priority Country: SE

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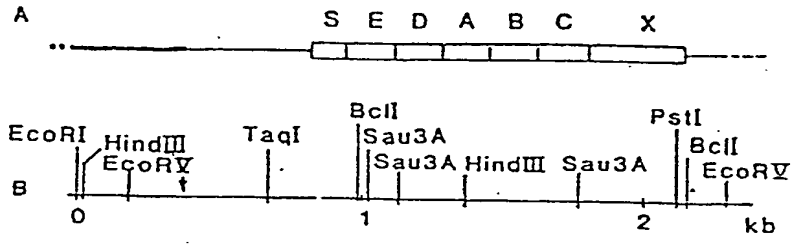
(81) Designated States: DE, JP, US.

Published
With international search report.

(54) Title: RECOMBINANT DNA MOLECULE, TRANSFORMED MICROORGANISM AND PROCESS FOR PRODUCING STAPHYLOCOCCAL PROTEIN A



(57) Abstract

A recombinant DNA molecule comprising at least one deoxynucleotide sequence coding for protein A or an active polypeptide fragment thereof and a process for preparing such a recombinant DNA molecule by introducing a deoxynucleotide sequence coding for protein A or an active fragment thereof into a cloning vehicle. Also a microorganism transformed by such a recombinant DNA molecule and its preparation are provided as well as a process for preparing protein A by culturing such a transformed microorganism in a suitable nutrient medium and isolating the product.

* (Referred to in PCT Gazette No. 14/1984, Section III)

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Recombinant DNA molecule, transformed microorganism and process for producing staphylococcal protein A.

The present invention relates to recombinant deoxyribonucleic acid which codes for protein A or an active derivative thereof as well as a process for the preparation thereof. The invention also relates to a transformant microorganism which includes such recombinant deoxyribonucleic acid, the preparation thereof and the use thereof for the production of protein A or an active derivative thereof.

Protein A is a cell wall component of the bacterium Staphyloccocus aureus, hereinafter called S. aureus, and is characterized by a specific serological reaction with mammal immunoglobulins. In contrast to the normal antigen-antibody reactions, however, protein A binds to the Fc-portion of all subclasses of human immunoglobulin type G, or IgG, except IgG_3, leaving the Fab-portion thereof free for antigen and hapten coupling. This property has given protein A a widespread use in both quantitative and qualitative immunochemical techniques. Covalently bonded to a carrier protein A is thus an excellent immunosorbent for the isolation of IgG. Moreover, complexing of protein A to IgG has been shown to activate the complement system, and recently protein A has been tested in the treatment of cancer and immunocomplex disorders by removing IgG complexes from the blood plasma therewith.

Protein A, whose exact structure may vary depending on its origin, has a reported molecular weight of about 42,000 and a markedly extended shape. Sequence analysis has revealed two functionally and structurally different regions of the molecule. The N-terminal part with a molecular weight of 27,000 consists of four or five consecutive and highly homologous IgG-binding units, each having a molecular weight of about 7,000. The C-terminal part of the protein, which has a molecular weight of about 15,000, is a region covalently bound to the peptidoglycan moiety of the cell wall and does not have any Fc-binding ability. A proposed primary structure for the Fc-binding portion of S. aureus protein A is shown in Sjödahl, J., Eur. J. Biochem. 73, 343-351 (1977) and 78, 471-490 (1977).

The biosynthesis of protein A takes place during the exponential growth phase. Most of the protein is bound to the cell wall, but in the stationary growth phase there is always some release due to autolysis. Protein A can be liberated from such S. aureus strains by digestion with the enzyme lysostaphin and subsequent affinity chromatography on agarose having IgG bound thereto. There are also strains of S. aureus which are unable to incorporate protein A into the

cell wall and thus all produced protein A is sectreted into the growth medium. Such extracellular production of protein A is common among methicillin resistant strains and, of course, facilitates the isolation of the protein A.

Production of protein A from strains of S. aureus has, however, two major disadvantages. One is the pathogenic nature of the bacteria requiring special safety precautions in the cultivation process, and the second is the relatively low quantities of protein A produced by the currently used S. aureus strains. It would therefore be highly desirable to be able to use a microorganism capable of producing protein A, or an active derivative thereof, which microorganism on one hand, is less or preferably non-pathogenic and, on the other hand, has an increased production of protein A. Such a microorganism is provided by the present invention.

In accordance with the present invention, which relates to so-called genetic engineering, a recombinant DNA molecule is provided which includes a deoxyribonucleotide sequence containing the genetic information for protein A or an active derivative thereof, i.e. that is capable of binding at least one IgG Fc-portion thereto, or precursors thereto. Such a recombinant DNA molecule or so-called hybrid vector can be introduced into any suitable host microorganism, which can then be cultured to produce the desired protein A material. By suitably selecting the DNA transfer vector as well as the host microorganism, a transformed microorganism can be obtained, which, on one hand, will be non-pathogenic and, on the other hand, will produce substantially greater quantities of protein A than the hitherto used S. aureus strains due to self-replication of the vector DNA in the host cells producing an amplified quantity of genes coding for protein A.

The relatively new recombinant DNA technology or genetic engineering referred to above permits the introduction of a specific nucleotide sequence coding for a desired protein or polypeptide into a bacterial or other appropriate host cell thereby conferring the desired property thereto. The DNA may be prepared by chemical synthesis or extracted from another bacterial strain or other organism. The construction of such transformant microorganisms may comprise the steps of producing a double-stranded DNA sequence coding for the desired protein or polypeptide; linking the DNA to an appropriate site in an appropriate cloning vehicle or vector to form recombinant DNA molecules, some of which will contain the desired protein coding gene; transforming an appropriate host microorganism with the recombinant DNA molecules; screening the resulting clones for the presence of the protein or polypeptide coding gene by

suitable means; and selecting and multiplying one or more positive clones. Optionally, one or more re- or subclonings may be performed, comprising the extraction and cleavage of the protein coding DNA, insertion of the cleaved fragments into a second cloning vehicle or vector and screening for the presence of the protein or polypeptide coding gene.

Several bacterial as well as non-bacterial proteins have been obtained, using such recombinant DNA technology, mostly in Escherichia coli, usually called E. coli, and are described in the literature. None of the previously known recombinant DNA-processes is, however, directed towards the synthesis of protein A or protein A-like materials, as is the present invention. The production of protein A with the use of recombinant DNA technology further requires that a DNA sequence of at least partially unknown structure, i.e. the DNA sequence coding for protein A in a suitable host, is found in and separated from a highly complex mixture of DNA sequences, as will be further explained below. Once the DNA sequence has been identified the recombinant DNA technology offers the possibility of preparing microorganisms capable of producing not only protein A but also modified protein A products having protein A activity, such as fragments of protein A and oligomeric forms of protein A or the active fragments thereof, or combinations, as will be further described hereinafter.

Thus, one aspect of the present invention relates to a novel microorganism prepared by recombinant DNA technology, which microorganism is capable of producing protein A or an active derivative thereof as defined below, as well as the preparation of such a microorganism through transformation of a host organism with a recombinant DNA molecule.

Another aspect of the invention relates to such a recombinant DNA molecule, which comprises at least one DNA sequence coding for protein A or an active fragment thereof, and to a process for its preparation.

Still another aspect of the invention relates to a process for preparing protein A or an active derivative thereof by culturing a transformed microorganism of the invention.

In the present description and claims the term "protein A" means any protein macromolecule having analogous or similar structure and immunological and biological activities to the protein A substance produced by staphylococci, such as the natural strains of S. aureus, including any mutants thereof. Thus, structural variations between the various members may occur. The expression "active derivative" (of protein A)is meant to comprise any polypeptide fragment of protein A which is capable of binding at least one immunoglobulin at the Fc-

part thereof or a free Fc- fragment, as well as oligomeric forms of protein A or active polypeptide fragments thereof. Such oligomeric forms may comprise two or more linked protein A molecules, active polypeptide fragments or combinations thereof and may possess increased IgG-binding activity compared to the normal protein A molecule. The terms "protein A" and "active derivative" are further meant to comprise protein A molecules and active protein A derivatives, respectively, as defined above, which may have a non-protein A peptide sequence linked thereto, e.g. due to insertion of chemically synthetized oligonucleotides when constructing the cloning vehicle.

Further specific terms used in the following description (some of which already used in the foregoing part) are defined below;

Nucleotide — A monomeric unit of DNA or RNA consisting of a sugar moiety (pentose), a phosphate, and a nitrogenous heterocyclic base. The base is linked to the sugar moiety via the glycosidic carbon (1' carbon of the pentose) and that combination of base and sugar is a nucleoside. The base characterizes the nucleotide. The four DNA bases are adenine ("A"), guanine ("G"), cytosine ("C") and thymine ("T"). The four RNA bases are A, G, C and uracil ("U").

DNA sequence — A linear series of nucleotides connected one to the other by phosphodiester bonds between the 3' and 5' carbons of adjacent pentoses.

Codon — A DNA sequence of three nucleotides (a triplet) which encodes through messenger RNA ("mRNA") an amino acid, a translational start signal or a translational termination signal. For example, the nucleotide triplets TTA, TTG, CTT, CTC, CTA and CTG encode for the amino acid leucine ("Leu"), TAG, TAA and TGA are translational stop signals and ATG is a translational start signal.

Plasmid — A non-chromosomal double-stranded DNA sequence comprising an intact "replicon" such that the plasmid is replicated in a host cell. When the plasmid is placed within a unicellular host organism, the characteristics of that organism are changed or transformed as a result of the DNA of the plasmid. For example, a plasmid carrying the gene for tetracycline resistance (Tet) transforms a host cell previously sensitive to tetracycline into one which is resistant to it. A host cell transformed by a plasmid is called a "transformant".

Phage or Bacteriophage — Bacterial virus many of which include DNA sequences encapsidated in a protein envelope or coat.

Cloning Vehicle — A plasmid, phage DNA or other DNA sequence which is able to replicate in a host cell, characterized by one or a small number of endonuclease recognition sites, or restriction sites, at which its DNA sequence may be cut in a determinable fashion without attendant loss of an essential

biological function of the DNA, e.g., replication, production of coat proteins or loss of promoter or binding sites, and which contains a marker suitable for use in the identification of transformed cells, e.g., tetracycline resistance or ampicillin resistance. A cloning vehicle is also known as a vector.

Host — An organism which on transformation by a cloning vehicle enables the cloning vehicle to replicate and to accomplish its other biological functions, e.g., the production of polypeptides or proteins through expression of the genes of a plasmid.

Cloning — The process of obtaining a population of organisms or DNA sequences derived from one such organism or sequence by asexual reproduction.

Expression — The process undergone by a gene to produce a polypeptide or protein. It is a combination of transcription and translation.

Transcription — The process of producing mRNA from a gene.

Translation — The process of producing a protein or polypeptide from mRNA.

Promoter -- The region of the DNA of a gene at which RNA polymerase binds and initiates transcription. A promoter is located before the ribosome binding site of the gene.

Ribosome Binding Site -- The region of the DNA of a gene which codes for a site on mRNA which helps the mRNA bind to the ribosome, so that translation can begin. The ribosome binding site is located after the promoter and before the translational start signal of the gene.

Gene — A DNA sequence which encodes, as a template for mRNA, a sequence of amino acids characteristic of a specific polypeptide or protein. A gene includes a promoter, a ribosome binding site, a translational start signal and a structural DNA sequence. In the case of an exported or secreted protein or polypeptide, the gene also includes a signal DNA sequence.

Expression Control Sequence -- A DNA sequence in a cloning vehicle that controls and regulates expression of genes of the cloning vehicle when operatively linked to those genes.

Signal DNA Sequence -- A DNA sequence within a gene for a polypeptide or protein which encodes, as a template for mRNA, a sequence of hydrophobic amino acids at the amino terminus of the polypeptide or protein, i.e., a "signal sequence" or "hydrophobic leader sequence" of the polypeptide or protein. A signal DNA sequence is located in a gene for a polypeptide or protein immediately before the structural DNA sequence of the gene and after the translational start signal (ATG) of the gene. A signal DNA sequence codes for the

signal sequence of a polypeptide or protein which (signal sequence) is characteristic of a precursor of the polypeptide or protein.

Precursor — A polypeptide or protein as synthetized within a host cell with a signal sequence.

Downstream and Upstream -- On a coding DNA sequence downstream is the direction of transcription, i.e. in the direction from 5' to 3'. Upstream is the opposite direction.

Recombinant DNA Molecule or Hybrid DNA -- A molecule consisting of segments of DNA from different genomes which have been joined end-to-end outside of living cells and have the capacity to infect some host cell and be maintained therein.

Structural DNA Sequence -- A DNA sequence within a gene which encodes, as a template for mRNA, a sequence of amino acids characteristic of a specific mature polypeptide or protein, i.e., the active form of the polypeptide or protein.

A basic aspect of the invention is the provision of a recombinant DNA molecule comprising a deoxynucleotide sequence coding for protein A, an active derivative of protein A or precursors thereto. The origin of said deoxynucleotide sequence is not critical to the invention, but any source can be used, including natural, synthetic or semi-synthetic DNA sources. In practice, however, the source of DNA coding for protein A will be a bacterial donor, viz. a protein A producing Staphylococcus species, such as a strain of S. aureus. Any such protein A producing staphylococcal donor may be used for the purposes of the present invention. For the active polypeptide fragments of protein A, and possibly also for the whole protein A molecule, however, synthetically produced molecules might be contemplated.

When preparing a recombinant DNA molecule in accordance with the present invention a suitable cloning vehicle or vector may be cleaved by means of a restriction enzyme and the DNA sequence or fragment coding for the desired protein A or active protein A derivative or precursors thereof inserted into the cleavage site to form a recombinant DNA molecule. This general procedure is known per se and various techniques or methods to link the DNA sequence to the cleaved cloning vehicle are described in the literature.

If staphylococcal chromosomal DNA is used as the source of the deoxynucleotide sequence to be inserted into the selected vector, it may be obtained by treating the Staphylococcus strain with the enzyme lysostaphin to form protoplasts, which are then lysed and the DNA extracted and isolated to form a

DNA preparation. By partial digestion with a suitable restriction enzyme the DNA preparation can be cleaved into fragments of an appropriate size. After treatment of the vector with the same or another restriction enzyme the vector cleavage products and the above fragmented DNA preparation are admixed and randomly combined under the ligating action of a ligase enzyme. Screening of the ligated DNA fragment combinations may be effected by making them biologically active in a suitable microorganism cell, e.g. a bacteria. Such transformation is another aspect of the present invention and will be described further below. The transformed cells, which contain either a vector or a vector/staphylococcal DNA combination, can be selected on the basis of a suitable marker included in the vector, e.g. antibiotic resistance, such as ampicillin resistance, which is not affected by insertion of the donor chromosomal DNA. Among the obtained clones recombinants can be recognized on the basis of a second marker included in the vector, e.g. tetracycline resistance, which is affected by the insertion of donor DNA and thus indicative of a transformed cell. In this way a "gene bank" of the S. aureus strain can be obtained, consisting of a great number, e.g. several hundreds, of clones which contain staphylococcal DNA. The clone or clones containing the protein A producing gene can then be found by any suitable assay for protein A, e.g. through ELISA technique (enzyme-linked immunosorbent assay). As mentioned above fragments of the DNA of protein A producing clones may be subcloned into the same or another host microorganism.

The cloning vehicles or vectors that can be used in accordance with the present invention depend i.a. on the nature of the host cell to be transformed, i.e. whether it is a bacterium, yeast or other fungi, etc. Useful cloning vehicles may, for example, consist of segments of chromosomal, non-chromosomal and synthetic DNA sequences, such as various known bacterial plasmids, e.g., plasmids from E. coli including pBR 322 and their derivatives, phage DNA, such as derivatives of phage lambda, vectors derived from combinations of plasmids and phage DNAs, yeast plasmids, specially constructed composite plasmids, etc. The particular selection of cloning vehicle with regard to a particular host may be made by a person skilled in the art. Also the site for the insertion of the DNA within each specific cloning vehicle may be selected by the skilled person, the cleavage sites depending on the restriction enzyme used. Of course, it is not necessary for a cloning vehicle useful in this invention to have a restriction site for insertion of the chosen DNA fragment, but the cloning vehicle could instead be joined to the fragment by alternative means, which are wellknown in the art.

Useful hosts for the purposes of the present invention may include

bacterial hosts, such as strains of Escherichia, Bacillus and Staphylococcus, e.g. E. coli, Bacillus subtilis and S. xylosus, yeasts and other fungi, plant cells in culture or other hosts. Among bacterial hosts those of the gram-positive type are preferred for the purposes of the present invention due to their less complex cell wall, which contains only one membrane system and thus permits secretion of the produced protein A material therethrough, as will be explained further below. Due to their non-pathogenic nature, e.g., the soil-bacterium Bacillus subtilis and Staphylococcus xylosus are considered as particularly useful as a final host in the present invention. It is, however, also within the scope of the present invention to transform already protein A producing strains of, e.g., S. aureus with the protein A coding recombinant molecule of the invention to multiply the number of protein A coding genes in the S. aureus cells. Yeast (whose genetics is fairly well known) is also a preferred final host to be transformed into a protein A producing microorganism in accordance with the present invention.

In a preferred embodiment of the recombinant DNA molecule of the present invention the DNA sequence coding for the desired protein A product is linked to a leader or signal sequence coding for a signal peptide. Such signal peptide, which will form an extension of the amino-terminal end of the desired protein or polypeptide product, is required for the transformed microorganism cell to secrete the synthetized product through its membrane. The product thus synthetized within the cell through expression of the inserted extrachromosomal gene will then be a precursor of the desired product. During the secretion process through the cell wall the signal peptide is cleaved off and only the mature protein A or active derivative thereof will be secreted into the surrounding medium. A microorganism transformed with such a signal sequence containing gene will thus not only synthetize the protein A or active derivative thereof but also secrete it into the culture medium. Hereby the necessity to rupture the cells to recover the protein A product is obviated and the isolation thereof is facilitated and made more efficient permitting a product of improved purity to be obtained. A number of signal peptides, as well as the corresponding DNA sequences, for both gram-positive and gram-negative bacteria as well as for yeasts are known and may be used in the present invention.

In the present invention it is most convenient - at least as far as bacterial hosts are concerned - to use the signal DNA sequence of the protein A gene as obtained from the staphylococcal strain, such as S. aureus, which is used as donor DNA sequence. In this case the DNA sequence coding for the corresponding protein A product precursor, i.e. the desired protein A product

fused to its signal peptide, is inserted into a cloning vehicle to prepare the recombinant DNA molecule of the invention. If, however, it is desired to use another signal sequence which is more functional in a particular host, such a signal sequence may be inserted into the cloning vehicle used in the invention by per se well-known methods which will not be described here.

Preferably also the promoter sequence of the donor staphylococcal strain is used and may be inserted into the cloning vehicle together with the signal sequence and the protein A coding sequence, which, e.g., will be the case when the whole protein A coding gene, from promoter to the stop codon, of the staphylococcal strain is inserted into the cloning vehicle. Other promoters may, of course, also be used, such as the promoter of a naturally occurring vector or of a vector modified by the insertion of strong external promoter.

DNA coding for an active polypeptide fragment of protein A as defined above may be obtained by introducing a DNA sequence comprising a suitable stop codon into the protein A coding gene by per se well-known methods. The stop codon may, e.g., be inserted such that only the portion of the gene that codes for the IgG-binding activity is translated. Expression of the gene will then produce a polypeptide corresponding to the IgG-active part of the protein A molecule.

DNA coding for oligomeric forms of protein A or IgG-active polypeptide fragments of the protein A molecule may be obtained by combining DNA fragments coding for protein A or IgG binding activity into a combined gene coding for such a dimer, trimer etc. Expression of the resulting gene will thus produce a protein A oligomer having increased IgG-binding activity compared to the normal protein A molecule. Methods for effecting such combination of DNA fragments are known per se and will not be described here.

The protein A or polypeptide product produced according to the present invention can be recovered by conventional techniques. When, e.g., the product is secreted through the cell membrane, which is the preferred embodiment, product isolation may be effected with conventional immunosorbent methods. If the product is trapped in the periplasmic space between two cell membranes, as in gram-negative bacteria, an osmotic shock procedure may be used to release the product into the growth medium, where it can be isolated as above. When, finally, the protein or polypeptide product is retained within the cell, as is the case when no signal peptide for the product is synthetized, the cells must be ruptured before, e.g., immunosorbent isolation, can be effected. Such rupture of the cell walls may, e.g., be done by pressing, ultrasonication, homogenization, shaking with glass-beads, etc.

The invention will now, by way of illustration only, be described in more detail in the following non-limiting examples, showing the cloning into E. coli and different Staphylococcus strains of the protein A gene from a staphylococcal strain with cell-wall associated protein. Reference will be made to the accompanying drawings.

In the drawings:

Fig. 1 is a schematic illustration of a circular restriction map of a plasmid DNA (pSPA1) coding for protein A. The size of the map is given in kilobases starting at the Eco RI restriction site at 12 o'clock, which is a restriction site within the vector pBR322. The positions of the Eco RI, Eco RV, Hind III, Pst I and Bam HI restriction sites are indicated. The junctions between the vector and the inserted DNA are indicated with arrows.

Fig. 2A is a schematic illustration of the protein A coding gene indicating its different regions. Heavy line represents the DNA of the vector pBR322. S is a signal sequence, A-D are IgG-binding regions previously identified, E is a region homologous to A-D, and X is the C-terminal part of protein A which lacks IgG-bindning activity.

Fig. 2B is a detailed restriction map of the DNA sequence corresponding to Fig. 2A and showing the restriction sites for Taq I, Hind III, Eco RV, Pst I, Bcl I and Sau 3A. The size is given in kilobases starting at the same Eco RI restriction site as indicated in Fig. 1. The junction between the vector pBR322 and the inserted DNA fragment is indicated with an arrow. The restriction sites for Taq I (two) and Rsa I (one) within the vector sequences have been omitted.

Fig. 3A-D show the base sequence for the structural protein A gene. Two possible promoters (-35 and -10) and a possible Shine-Dalgarno sequence (indicated by "=") are indicated. The amino acid sequence as deduced from the DNA sequence is also shown (the IUPAC amino acid abbreviations are used; J. Biol. Chem. 241, 527 and 2491 (1966)). The five amino acids (residues 99, 101 120, 199 and 273) that differ compared to the amino acid sequence reported by Sjödahl supra are indicated as well as the 8 residues (out of 50) in the region E which differ from the corresponding amino acid of region D. The start residues of regions S, E, D, A, B, C and X are indicated by arrows.

Fig. 4 is an autoradiograph of a nucleotide sequence gel showing the junction between regions D and E of Fig. 3. The sequencing (according to Maxam et al, P.N.A.S. 74, 560-564 (1977)) was performed on a DNA fragment labelled at the Bcl I site at position 0,9 kb in Fig. 2. The partially chemically degraded products were resolved in an 8% polyacrylamide sequencing gel (Maizel et al, Methods in Vir. 5, 179-246 (1970)).

Fig. 5 shows SDS-polyacrylamide gel electrophoresis of IgG-Sepharose® purified cell extracts. pBR322 and pSPA1 represent extracts of E. coli cells carrying the respective plasmid. SPA is commercially available protein A from S. aureus (Pharmacia, Uppsala, Sweden). Adeno 2 (AD 2) proteins were used as size markers.

Fig. 6 is a schematic map illustration of plasmid pSPA15, AMP and CML representing genes coding for ampicillin and chloramphenicol resistance, respectively, Ori being replication origins and SPA designating the structural protein A gene.

Fig. 7 is a schematic illustration of the constructions of plasmids containing the whole or parts of the protein A gene. A few restriction sites are shown. Boxes represent structural genes and the arrows indicate the orientation (from start codon towards stop codon). The replication origin is also indicated by Ori. AMP and TET are the genes coding for ampicillin and tetracycline resistance, respectively. PROT A is the gene coding for protein A and lac Z' is the gene coding for the N-terminal part of β-galactosidase. (Rüther et al, Nucl. Acids Res. 9, 4087-4098 (1981)).

Fig. 8 is a schematic illustration of the construction of plasmid pSPA16. The abbreviations used are the same as in Fig. 6. S, E, D and B are as in Fig. 2 and A' and C' represent parts of the respective IgG-binding regions A and C of the protein A coding gene.

Fig. 9 is a presentation of the nucleotide sequence, and the corresponding deduced amino acid sequence, around the 3'-end of the protein A gene in plasmid pSPA16. x x x represents the new stop codon.

Fig. 10A-C is a combined illustration, similar to Fig. 2, of the plasmids pSPA15 (B) and pSPA16 (C) together with a corresponding restriction map (A) aligned therewith. Heavy line represents the protein A structural gene.

Fig. 11 is a schematic illustration of the sequencing strategy (C) used for sequencing the 1.8 kb Taq I-EcoRV DNA fragment (B), containing the protein A coding gene (A), to obtain the sequence shown in Fig. 3.

In the Examples the starting materials, buffers, cell media and routine method steps were as follows.

STARTING MATERIALS

Bacterial hosts. Four strains of E. coli K12 were used in the Examples: HB101, described by Boyer et al, J. Mol. Biol. 41, 459-472 (1969); 259, described by Jacob, F. and Wollman, E.C. Ann. Inst. Pasteur 91, 486-510 (1956); GM 161, described by Marinus, M.G., Molec. gen. Genet. 127, 47-55 (1973); RRI del M15 (Langey et al, Proc. Natl. Acad. Sci., USA, 72, 1254-1257 (1975)).

(the strains are available at the Department of Microbiology (N), Biomedical Centre, Uppsala, Sweden).

Also, the following four Staphylococcus strains were used:

S. epidermidis 247, described by Rosendorf et al, J. Bacteriol. 120: 679-686 (1974); obtained from Inst. of Medical Microbiology, Univ. of Zürich, Switzerland;

S. xylosus KL117, described by Schleifer et al, Int. J. Syst. Bacteriol. 25: 50-61 (1975) and Schleifer et al, Arch. Microbiol. 122: 93-101 (1979); obtained from Inst. for Microbiology, Technical Univ. of Munich, Federal Republic of Germany;

S. aureus SA113, described by Iordanescu et al (J. Gen. Microbiol. 96: 277-281 (1976));

S. aureus 320, a protein A negative mutant of strain S. aureus 113 isolated at the Department of Microbiology, Biomedical Centre, Uppsala, Sweden and described by Jonsson et al, Curr. Microbiol. 8: ..... (1983).

Cloning vehicles. The cloning vehicles used in the Examples were pBR322 as constructed and described by Bolivar et al, Gene 2, 95-113 (1977); pBR328 as constructed and desribed by Soberon, X., et al, Gene 9, 287-305 (1980); pTR262 as constructed and described by Roberts, T.M., et al, Gene 12, 123-127 (1980); pHV14 as constructed and described by Ehrlich, S.D., Proc. Natl. Acad. Sci. USA 70, 3240-3244 (1978), and pHV33 as constructed and described by Primrose, S. B. and Ehrlich, S.D., Plasmid 6, 193-201 (1981). pUR222 as constructed and described by Rüther et al, Nucl. Acids Res., 9, 4087-4098 (1981);

BUFFERS AND MEDIA

Triton-mix:	0.1% Triton X-100, 0.125 M EDTA and 20 mM Tris (pH 8.0)
Tris-EDTA buffer ("TE"):	0.001 M EDTA and 0.01 M Tris (pH 7.8)
CY broth:	Difco casein hydrolysate 1%, Difco yeast extract 1% and glucose 0.5%; 4 ml of 1.5M glycerol-phosphate is added to 100 ml of CY broth
Coating buffer (carbonate-bicarbonate - pH 9.6):	1.59 g Na_2CO_3, 2.93 g $NaHCO_3$ and 0.2 g NaN_3, made up to 1 liter with distilled H_2O
PBS TWEEN (Phosphate buffered saline plus 0.05% TWEEN®):	8.0 g NaCl, 0.2 g KH_2PO_4, 2.9 g Na_2HPO_4 x 12 H_2O, 0.2 g KCl, 0.5 ml TWEEN® 20 and 0.2 g NaN_3, made up to 1 liter with distilled H_2O; pH 7.4

Diethanolamine buffer 10%:	97 ml dietyhanolamine, 800 ml distilled H_2O, 0.2 g NaN_3, and 100 mg $MgCl_2 \times 6\ H_2O$; pH adjusted to 9.8 with 1 M HCl; made up to 1 liter with distilled H_2O
Luria-broth ("LB"):	10 g Difco tryptone, 5 g Difco yeast extract, 0.5 g NaCl, 2 ml 1 M NaOH; adjusted to pH 7.0 with 1 M NaOH; 10 ml 20% glucose added after autoclaving.
LA-medium	Luria-broth supplemented with 1% Difco agar
TEB buffer:	0.09 M Tris-borate, 0.09 M boric acid and 0.002 M EDTA

ROUTINE METHODS

Certain procedures were carried out repeatedly in the Examples. Unless otherwise specified, they were done exactly as follows each time that they were carried out.

Transformations. Transformation of E. coli K12, with plasmid DNA, was performed exactly as described by Morrison, D.A., Methods in Enzymology, Academic Press 68, 326-331 (1979). The transformed cells were selected in a conventional manner on plates by plating for single colonies on LA plates containing suitable antibiotics, i.e. 35 µg/ml of ampicillin or 25 µg/ml of chloroamphenicol.

Isolating plasmids. Large scale plasmid preparation was performed exactly as described by Tanaka, T. and Weisblum, B., J. Bacteriol. 121, 354-362 (1975). For scoring a large number of clones for plasmids the "mini alkali method" was used exactly as described by Birnboim, H.C. and Doly, J., Nucl. Acids Res. 7, 1513-1523 (1979).

Restriction enzyme digestion of DNA. DNA was cleaved with conventional restriction enzymes purchased from New England Bio Labs, Waltham MA, USA. The restriction enzymes were added to DNA at conventional concentrations and temperatures and with buffers as recommended by New England Bio Labs.

Ligating DNA fragments. All DNA fragments were ligated at 14°C over-night with T4 DNA ligase purchased from New England Bio Labs, Waltham, MA., USA, in a buffer recommended by the supplier.

Agarose gel electrophoresis. 0.7% agarose gel electrophoresis for separating cut plasmid fragments, supercoiled plasmids, and DNA fragments 1000 to 10,000 nucleotides in length was performed exactly as described by Helling et al, J. Vir. 14, 1235-1244 (1974).

Polyacrylamide gel electrophoresis. 5% polyacrylamide gel electrophoresis for the separation of DNA fragments 100 to 4000 nucleotides in length was performed exactly as described by Maxam et al, P.N.A.S. 74, 560-564 (1977). 13% polyacrylamide gel electrophoresis for the separation of proteins of molecular weights of 5,000 to 120,000 was performed exactly as described by Maizel et al, Methods in Vir. 5, 179-246 (1970).

Gel elution. DNA fragments were eluted from either polyacrylamide or agarose gel pieces exactly as described by Maxam et al, P.N.A.S. 74, 560-564 (1977).

DNA sequencing. DNA fragments were 5' end labeled, and their DNA sequences were determined exactly as described by Maxam et al, supra. The 5' end of endonuclease generated DNA fragments was labelled with (γ-^{32}P) ATP (New England Nuclear, USA; 2700 Ci/mmol) using T4 polynucleotide kinase (Boehringer, Mannheim, West Germany).

Preparation of cell lysate for detection of protein A. E. coli clones were grown overnight at 37°C in 50 ml Luria-broth (LB) with ampicillin added at 35 µg/ml. After centrifugation the cells were resuspended in 5 ml Tris-EDTA (0.05 M, pH 8.5, 0.05 M) and centrifuged. The cells were resuspended in 5 ml of the same buffer and lysozyme was added to a final concentration of 2 mg/ml. After 1 hour at 37°C the lysate was centrifuged in a Sorvall SS-34 rotor at 15,000 rpm for 15 minutes. The supernatant was collected and assayed for protein A.

Detection and quantification of protein A from E. coli clones.

An ELISA-test (enzyme linked immunosorbent assay) was used for detection and quantification of produced protein A. The test makes use of a special microtiter plate (Titertek, Amstelstad, the Netherlands) having no net charge (neutral), the wells of which are coated with human IgG (Kabi, Sweden). Test samples are then added to allow protein A to bind to the Fc-part of the IgG-molecules. The amount of remaining free Fc-sites is then titrated by adding alkaline phosphatase linked to protein A. After washing of the wells, p-nitrophenyl-phosphate is added as a substrate for alkaline phosphatase.

Assay: The wells of a microtiter plate were filled with 50 µl of a solution of human IgG (Kabi, Sweden) at 500 µg/ml in a coating buffer and the plate was

incubated at room temperature for 1 hour. The wells were then washed three times with PBS +0.05% Tween® 20, which was used in all washes in the assay, and 50 µl of the lysate to be tested was added. For quantitative determinations twofold serial dilutions of the lysates in PBS+0.05% Tween® 20 were made. 10 µl of PBS+0.1% Tween® 20 was then added and incubation was allowed for 1 hour at room temperature. The wells were again washed three times, and 50 µl of protein A-alkaline phosphatase conjugate (prepared exactly as described in Immunochemistry, Pergamon Press 1969, Vol. 6 pp. 43-52) was added. After 1 hour of incubation at room temperature the wells were again washed three times and 100 µl of alkaline phosphatase substrate (Sigma 104 = p-nitrophenyl-phosphate at 1 mg/ml) was added. The enzyme reaction was interrupted after 30 minutes by the addition of 10 µl of 3 M NaOH. The result was determined visually. A positive result, i.e. presence of protein A, is a colour-less reaction mixture, since no free Fc-sites of IgG are available to bind the conjugate. A negative result, i.e. no protein A, is observed as a yellow colour due to the activity of the alkaline phosphatase of the bound conjugate. Quantitative determinations of protein A were made by running serial twofold dilutions of a protein A standard solution of known concentration in parallel with the test samples.

EXAMPLE I

Cloning of protein A in E. coli

A. Preparation of staphylococcal chromosomal donor DNA. S. aureus strain 8325-4 (Ø11) mec-4916, str-4916, nov-142 (described by Sjöström, J.-E., et al, J. Bacteriol. 123, 905-915 (1975) and available from the Department of Microbiology (N), Biomedical Center, Uppsala, Sweden) was grown to OD_{540}=0.2 in Cy broth. One litre of cell culture was harvested by centrifugation at 5,000 rpm in a Sorvall GSA rotor, resuspended in 100 ml of 0.9% NaCl and 10 mM Tris, pH 7.2, and centrifuged at 5,000 rpm in a Sorvall GSA rotor. The cell pellet was finally resuspended in 10 ml of 25% sucrose, 50 mM Tris pH 7.2 and protoplasts were prepared by lysostaphine treatment (15 µg/ml) at 37°C for 30 min. The protoplasts were lysed by addition of 10 ml of Triton-mix and 5 ml of H_2O. The mixture was left on ice and occasionally gently shaken until complete lysis. The DNA was treated with proteinase K (0.1 mg/ml) and SDS (sodiumdodecyl sulfate) (0.5%) for 1 hr at 37°C followed by five phenol extractions with equal volumes of phenol, and finally two chloroform extractions. Sodium acetate, pH 7.0, was added to 0.3 M, and the DNA was precipitated with two volumes of cold ethanol. The precipitate was washed stepwise in 70, 80, 90 and 99% cold ethanol. The

precipitate was dissolved in TE buffer by gentle mixing at 37°C. Finally the DNA was dialysed against TE buffer.

B. Partial digestion of chromosomal DNA and isolation of donor fragments. Purified staphylococcal DNA from step A was digested with various concentrations of the restriction enzyme Mbo I. Each reaction was made in 50 µl volume with 1 µg of DNA and the reaction was stopped by heat inactivation at 65°C for 10 min. The extent of digestion was determined by agarose gel electrophoresis. The concentration of Mbo I giving a large partial cleavage product of 5 to 20 kilobases was chosen for a preparative digest of 100 µg of staphylococcal DNA in 5 ml. This digest was heat inactivated, precipitated with ethanol, dissolved in 100 µl of TE and sedimented through a 10-30% sucrose gradient in TE buffer. A Beckman Sw40 rotor was used at 5°C, 35 rpm, for 20 hrs. The gradient was fractioned into 0.5 ml fractions, each of which was analyzed by agarose gel electrophoresis. The fractions with 8-10 kb fragments were pooled, precipitated with 2 volumes of ethanol and dissolved in TE buffer.

C. Digestion and alkaline phosphatase treatment of the vector pBR322. One µg°of pBR322 was digested with Bam HI for 2 hrs at 37°C, and the enzyme was inactivated at 65°C for 10 minutes. The DNA was treated with alkaline phosphatase in order to remove the 5' phosphate. This treatment eliminated the possibility to re-ligate the vector. The reaction was effected in 50 mM Tris, pH 7.9, 5% DMSO and 1 unit of calf intestinal alkaline phosphatase at 37°C for 30 minutes in 1 ml. 0.5% SDS was added and the DNA was phenol extracted twice. Traces of phenol were removed by ether and the DNA was precipitated by two volumes of ethanol.

D. Insertion of staphylococcal DNA into pBR322, transformation of E. coli and negative selection for recombinants. The vector pBR322 chosen for the original cloning of staphylococcal DNA codes for tetracycline (tet) and ampicillin (amp) resistance. When pBR322 is opened up by digestion with Bam HI, as in step C above, and a DNA fragment is inserted, the gene for tetracycline resistance is inactivated. By testing transformants for sensitivity to tetracycline recombinants can be found - so-called negative selection. 0.5 µg of pBR322 treated according to Step C and 2 µg of staphylococcal DNA treated according to Step B were mixed and ligated in a total volume of 25 µl overnight at 14°C. The mixture was used to transform E. coli 259 with selection for ampicillin resistance (35 µg/ml). Transformants were picked and streaked on plates containing 10 µg/ml of tetracycline and plates containing 35 µg/ml of ampicillin, respectively. Transfor-

mants that grew on ampicillin but not on tetracycline were considered as recombinants.

E. Detection of protein A positive E. coli clones.
Five hundred tetracycline sensitive clones from Step D were grown as separate colonies on LA-plates (prepared from LA-medium) containing ampicillin (35 µg/ml). Groups of 25 colonies were collected and inoculated into 50 ml of LB-broth with 35 µg of ampicillin and grown overnight. Cell extracts were prepared by lysozyme+EDTA treatment (as described under Routine Methods) and tested for protein A by the ELISA-test described under Routine Methods. One of these groups of clones was positive, and this positive group was further subdivided into 5 groups of 5 clones each and grown and treated as above. Finally in a last series of tests one protein A producing clone, E. coli SPA 11 containing the plasmid pSPA1, was found. Cultures of this clone have been deposited with the collection of the Deutsche Sammlung von Mikroorganismen (DSM), Grisebachstrasse 8, 3400 Göttingen, Federal Republic of Germany, on July12, 1982 where it was assigned No. DSM 2434.

F. Restriction map of pSPA1. In order to get information for subcloning and sequencing the gene coding for protein A, a restriction map of pSPA1 obtained in Step E was made. This was done with single, double and/or triple digests with the enzymes indicated in Figs. 1 and 2. Fig. 2 shows a more detailed map in the area coding for protein A. Summing up the sizes of the various restriction fragments gives a total size of 12 kb for pSPA1, and thus the donated staphylococcal fragment amounts to approximately 7.6 kb.

G. Subcloning of the protein A coding gene from pSPA1 into plasmids pBR328 and pHV14. In order to locate the position of the gene several subclones were constructed and tested for protein A activity. 2 µg of plasmid pSPA1 from Step E and 1 µg of pBR328 were cut with the restriction enzyme Eco RV, mixed, treated with T4-ligase and used to transform E. coli HB101. Cleavage, ligation and transformation were realized as described above under Routine Methods. Colonies containing recombinants were selected as being chloramphenicol resistant and tetracycline sensitive in analogous manner as described in Step D. 8 colonies out of 48 of these recombinants were discovered to be protein A positive using the ELISA method described under Routine Methods. Restriction analysis, according to Step F, showed that all 8 clones contained pBR328 having a 2.15 kb Eco RV insert derived from the fragment corresponding to 0.2 kb to 2.35 kb of the pSPA1 restriction map of Fig. 1. All clones had the insert in the same

orientation giving a functional tet promoter reading into the inserted gene.

One clone containing this plasmid, designated pSPA3, was selected for further studies. In order to determine whether the protein A gene could be transcribed from a promoter of its own, the plasmid pSPA3 was used as a source to reclone it into the plasmid pHV14. This plasmid, which is derived from pBR322, has a 2.8 kb insert in the Hind III restriction site, thus inactivating the tet-promoter. Any insert in the Eco RV restriction site of this plasmid therefore must have a functional promoter of its own in order to be transcribed by E. coli RNA polymerase. 1 µg of pSPA3 and 1 µg of pHV14 were cut with Eco RV, admixed, treated with T4-ligase and used to transform E. coli HB101. Cleavage, ligation and transformation were effected as described above. Colonies were selected as being ampicillin resistant and tetracycline sensitive as described in Step D.

Plasmids from 52 of these colonies were isolated by the "mini alkali method" referred to under Routine Methods and tested by running on 0.7% agarose gel electrophoresis. One of these clones was discovered to be a recombinant of pHV14 having the above mentioned 2.15 kb Eco RV insert from pSPA3. The clone containing this plasmid, designated pSPA5, was found to be protein A positive when tested using the ELISA method. It was concluded that the insert must contain a staphylococcal promoter reading into the protein A gene which also is functional in E. coli HB101.

Analysis of the DNA sequence of the protein A gene.

The results of the subcloning indicated that DNA-sequencing should be started at the Hind III-site at map position 1.4 kb going counter-clockwise (Fig. 1). The DNA source for the sequencing analysis was purified pSPA3. By comparing the partially known amino acid sequence of protein A (as reported by Sjödahl supra) with the obtained DNA sequence the position of the HIND III-site in the gene could be located. As shown in Figs. 2 and 3 this restriction site is within region A of protein A. By further analysis, according to Step F, restriction sites for the enzymes TaqI, Rsa I, Bcl I, Sau 3A and Pst I were determined (Fig. 2). These sites were used for sequencing in one or both directions giving in most cases nucleotide sequences of both strands. The strategy used for the sequencing is outlined in Fig. 11. As Bcl I does not cleave DNA purified from E. coli HB101, pSPA3 was transformed into the strain E. coli GM 161 lacking the enzyme D-alanine methylase. pSPA3 repurified from this strain was cleaved with Bcl I for sequencing.

Fig. 3 shows the DNA sequence of the whole staphylococcal protein A

gene. The amino acid sequence deduced from the DNA sequences is also indicated together with the differing amino acids as compared to the sequence proposed by Sjödahl supra, which was made on another strain of S. aureus (Cowan I).

The DNA sequence illustrated in Fig. 3 reveals an N-terminal region called E similar to the repetitive regions D-A-B-C reported by Sjödahl supra. This region of 50 amino acids has 42 amino acids which are identical to region D.

Region E is preceded by a leader sequence with the characteristics of a signal peptide containing a basic region of 11 amino acids followed by a hydrophobic stretch of 23 amino acids. The exact cleavage site is not known, but possible sites are at alanine residues 36, 37 or 42, probably at 36. If so, the amino acid sequence 37-42 belongs to region E of the protein A molecule. The initiation codon for translation is TTG similar to a few other reported initiation codons from gram-positive bacteria . Six nucleotides upstream TTG a Shine-Dalgarno sequence (defined in Shine, J. and Dalgarno, L. Nature (London) 254, 34-38 (1975)) is found which has many features in common with other gram-positive ribosome binding sites . Further upstream two possible promoters are found at -35 and -10 (Fig. 3).

By calculating the number of bases necessary to code for the whole protein it seems that both pSPA1 and pSPA3 contains the complete protein A structural gene.

Analysis of the gene product from E. coli containing pSPA1. E. coli cells carrying pSPA1 were grown overnight in 400 ml of LB with ampicillin, 35 µg/ml, added. The cell culture was centrifuged at 6000 rpm with a Sorvall GSA-rotor for 10 min. and the cell pellet was washed in 20 ml of TE (0.05 M, pH 8.5, 0.05 M EDTA) and again centrifuged as above. This time the cell pellet was resuspended in 15 ml of a protease inhibitor buffer (0.02 M potassium phosphate, pH 7.5, 0.1 M NaCl, 0.5% sodium deoxycholate, 1% Triton X-100, 0.1% sodiumdodecyl sulfate (SDS), and 1 mM phenylmethylsulfonyl fluoride (PMSF)). The cells were then sonicated in a MSE sonicator for 4x40 sec. on an ice-bath and centrifuged at 15,000 rpm (Sorvall SS-34 rotor) for 10 min. The supernatant was collected and passed over an IgG-Sepharose® 4B column (Pharmacia, Uppsala,Sweden) (Hjelm et al, FEBS Lett. 28, 73-76 (1972)) that had been equilibrated with a sodium acetate buffer (0.1 M sodium acetate, 2% NaCl, pH 5.5). The column was then washed with the same buffer as above and the adsorbed protein A eluted with a glycine buffer (0.1 M glycine, 2% NaCl, pH 3.0). To the eluted fractions 1/9 volume of 100% trichloroacetic acid (TCA) was added.

The samples were precipitated for 6 hours at +4°C and centrifuged at 12,000 rpm in an Eppendorf centrifuge for 15 min. The pellets were washed once in 1 ml of cold acetone and then centrifuged as above. The remaining pellets were dried, dissolved in TE and pooled to give a total volume of 400 µl.

The protein concentration was determined, and 20 µg were analyzed on a 13% SDS-polyacrylamide gel at 100 V for 12 hrs. The gel was stained with amidoblack (0.1%, 45% methanol, 10% acetic acid). An extract from cells carrying pBR322 was prepared in parallel and the same volume as above was analyzed on the gel. The results of the gel electrophoresis are shown in Fig. 5 indicating that protein A produced in E. coli carrying pSPA1 migrated close to pure protein A from S. aureus (from Pharmacia, Uppsala, Sweden). The extract from cells carrying the pBR322 plasmid had no corresponding protein.

Analysis of localization of protein A in E. coli

Enzymes, which in gram-positive bacteria are extracellular, are in gram-negative bacteria often located between the inner and outer membranes in the so-called periplasm or periplasmic space. Since protein A is located in the cell wall and thus outside the cell membrane in S. aureus, the localization of the protein in the transformed E. coli cells containing pSPA1 was determined. For this purpose the osmotic shock procedure as described by Heppel, L. A., Science 158; 1451-1455 (1967) was used. This procedure releases proteins from the periplasmic space but not intracellular enzymes. Alkaline phosphatase was used as an example of a protein found in the periplasmic space and phenylalanine-tRNA synthetase as an example of an intracellular protein.

E. coli containing pSPA1 was grown in a low phosphate medium (exactly as described by Neu, H.g. and Heppel, L.A., J. Biol. Chem. 240; 3685-3692 (1965)) to derepress the synthesis of alkaline phosphatase. One liter of an overnight culture (approximately 7.5×10^8 CFU/ml) was divided into two portions. One portion was washed three times in cold 0.01 M Tris-HCl buffer, pH 8.1, and the cells resuspended in 20 ml of 20% sucrose-0.03 M Tris-HCl, pH 8.1, 1mM EDTA. After 10 min. on a rotary shaker at room temperature the mixture was centrifuged for 10 min. at 13,000x g in a Sorvall centrifuge. The supernatant was removed and the well drained pellet was rapidly mixed with 20 ml of cold 5×10^{-4} M $MgCl_2$ solution. The suspension was mixed in an icebath on a rotary shaker for 10 min. and centrifuged. The supernatant termed "the osmotic shock wash" was collected for further testing. For comparison the other portion of cells was centrifuged, washed and resuspended in 5 ml of polymix-buffer (I) (exactly as described by Jelenc, P.C., Anal. Biochem. 105; 369-374 (1980)). The cells were

disintegrated in an X-press as recommended by the manufacturer (Biotec, Stockholm, Sweden). The cell debris were removed by centrifugation at 13,000 rpm for 15 min. in a Sorvall SS-34 rotor centrifuge and the supernatant was collected for further testing.

The two extracts obtained containing perisplasmic and whole cell protein, respectively, were each assayed for alkaline phosphatase and phenylalanine-tRNA synthetase with the enzymatic assays indicated below, and for protein A as described above under Routine Methods.

Enzymatic assays

Alkaline phospatase was assayed in a Tris-buffer, 0.05 M, pH 8.0, using p-nitrophenyl-phosphate ($4x10^{-4}$M) as substrate (Sigma 104). Hydrolysis of p-nitrophenyl-phosphate was measured in a spectrophotometer at 410 mμ. One unit of activity represented a change in absorbance at 410 mμ of 1.0 per minute. (Heppel, L.A., Harkness, D.R. and Hilmoe, R.J., J. Biol. Chem. 237, 841-846 (1962).

Phenylalanine-tRNA synthetase

The assay was performed in a mixture containing in a total volume of 100 μl: 5 mM $Mg(OAc)_2$, 0.5 mM $CaCl_2$, 95 mM KCl, 5 mM NH_4Cl, 8 mM putrescine, 1 mM spermidine, 5 mM K-phosphate, pH 7,5, 1 mM dithioerythritol, 1 mM ATP, 6 mM phosphoenolpyruvate, 1 μg pyruvate kinase (Sigma, St. Louis, USA), 1 unit of myokinase (Sigma), 100 μM (^{14}C)-phenylalanine (4 cpm/pmol) (Radiochemical centre, Amersham, England), 300 μg of total E. coli tRNA (Boerhringer/Mannheim, Federal Republic of Germany).

The X-pressed cell extract and the osmotic shock wash were tested by the addition of 10 μl of suitable dilutions. The enzyme assays were run for 15 min. at 37°C. Cold 10% TCA was added to interrupt the reaction and to precipitate phenylalanine-tRNA. The precipitate was collected on glass fibre filters, (GFA, Whatman), washed with 10% cold TCA and cold 70% ethanol and the radioactivity measured. One unit of activity is defined as the formation of 1 pmole of Phe-tRNA per minute. (Wagner, E.G.H., Jelenc, P.C., Ehrenberg, M. and Kurland, C.G., Eur. J. Biochem. 122,193-197 (1982)).

The results are presented in the following Table 1. All figures in the table are calculated per 500 ml cell culture (approx. $7.5x10^8$ CFU/ml).

TABLE I

Enzyme activities and protein A content of E. coli cells containing pSPA1

	Periplasm of cells	Whole cells
Alkaline phosphatase (units)	160	210
Phenylalanine-tRNA synthetase (units)	0	1530
Protein A (µg)[a]	24-48	24-48

[a]Since the determination is made in serial two-fold dilutions the amounts are presented as lying within the range of two dilution steps.

Table I shows that protein A and alkaline phosphatase were released when the cells were subjected to osmotic shock, while no activity of the intracellular enzyme phenylalanine-tRNA synthetase was detected in the osmotic shock wash. This result indicates that the S. aureus signal sequence, which according to the sequence data (Fig. 3) is present in the cloned DNA, is expressed in E. coli, and that the signal peptide is recognized by the membrane. In a gram-positive bacteria, lacking the outer membrane, such as e.g. Bacillus subtilis, the signal peptide would most likely have effected secretion of the protein A into the growth medium.

The amounts of protein A produced by the pSPA1-carrying E. coli cells are about 1-2 mg/liter medium.

EXAMPLE II

Cloning of protein A in various staphylococcal strains

I. Construction of shuttle vectors containing the protein A gene

Two shuttle vectors containing the protein A gene were constructed to enable replication both in E. coli, S. aureus and coagulase-negative staphylococci. The plasmid vector pHV33 based on the staphylococcal plasmid pC194, was used expressing ampicillin and tetracycline resistance in E. coli and chloramphenicol resistance in staphylococci.

A. Construction of shuttle vector plasmid pSPA15 (Fig. 6)

The first shuttle vector was constructed by cloning the 2,1 kb EcoRV fragment containing the protein A gene as described in Example I, step G, into the EcoRV site of plasmid pHV33. 2 µg of plasmid pSPA3, from step G of Example I, and 1 µg of pHV33 were cut with EcoRV, mixed, treated with T4-ligase and used to transform E. coli HB101. Cleavage, ligation and transformation

were performed as described above under Routine Methods. Colonies containing recombinants were selected as being ampicillin resistant and tetracycline and chloramphenicol sensitive in analogous manner as described in step D of Example I. Restriction analysis according to step F of Example I showed that out of 8 tested clones all contained the plasmid shown schematically in Fig. 6. All clones had the insert in the same orientation as in plasmid pSPA3 (see step G, Example I). One clone containing this plasmid, designated pSPA15, was selected for further studies. This plasmid was found to contain the whole structural gene of protein A coding for a mature protein of 447 amino acids and a predicted molecular weight of 49,604.

B. Construction of shuttle vector plasmid pSPA16 (Fig. 8)

B1 Subcloning of the 5'-end of the protein A gene from pSPA1 into plasmid pTR262 to obtain plasmid pSPA2 (Fig. 7)

1 µg of plasmid pSPA1 (see Fig. 1) from step E of Example I, and 1 µg of plasmid pTR262 were cut with restriction enzymes Hind III and Pst I, mixed, treated with T4-ligase and used to transform E. coli HB101. Cleavage, ligation and transformation were effected as described above under Routine Methods.

Plasmid pTR262 contains a lambda repressor gene which on expression inactivates the gene for tetracycline resistance. The lambda repressor gene has a Hind III site and insertion of a DNA sequence into the latter therefore inactivates the lambda repressor gene and activates the tetracycline resistance gene. Plasmid pTR262 thus permits positive selection for tetracycline resistant recombinants.

Colonies containing recombinants were thus selected as being tetracycline resistant. 1 colony out of 20 of these recombinants was discovered to be protein A positive using the ELISA method described hereinbefore under Routine Methods. Restriction analysis indicated that it contained vector plasmid pTR262 having a 2.1 kb protein A gene insert derived from the fragment corresponding to 0.0 to 2.1 kb of the pSPA1 restriction map of Fig. 1 and 2B. This plasmid was designated pSPA2 and is shown schematically in Fig. 7. It has a unique Pst I restriction site at the 3'-end of the protein A gene fragment which will be utilized in the following step B5.

B2 Preparation of a DNA fragment containing the protein A gene

100 µg of plasmid pSPA5 from step G of Example I were cut with restriction enzyme Eco RI for 1 hr at 37°C. This produced two DNA fragments, viz. the inserted DNA fragment containing the protein A gene (2.1 kb) between positions 0.2 kb and 2.3 kb in Fig. 2B and the vector pHV14 (7.2 kb). This digest was heat inactivated, precipitated with ethanol, dissolved in 100 µl of TE and

sedimented through a 10-30% sucrose gradient in TE buffer. A Beckman SW40 rotor was used at 5°C, 35,000 rpm, for 20 hrs. The gradient was fractionated into 0.5 ml fractions, each of which was analyzed by agarose gel electrophoresis. The fractions containing the 2.1 kb fragment were pooled, precipitated with 2 volumes of ethanol and dissolved in TE buffer. As appears from Figs. 2A and B the fragment contains, in addition to the whole protein A gene, an E. coli sequence derived from plasmid pBR322 and a staphylococcal gene residue.

B3 Preparation of a DNA fragment containing part of the protein A gene

5 µg of the purified 2.1 kb fragment from step B2 were cut with restriction enzyme Sau 3A for 1 hr at 37°C. The digest was run on a preparative 8% polyacrylamide gel electrophoresis in TEB buffer. The gel was stained with ethidium bromide (1 µg/ml) and a DNA fragment of approximately 600 base pairs was cut out. This fragment corresponds to the part of the gene between positions 1.15 and 1.8 kb in Fig. 2B. The DNA was eluted overnight at 37°C in 5 ml of TE + 0.3 M NaCl. The eluate was passed over a column containing approximately 300 µl of sedimented DE-52 (Whatman, England) equilibrated with 5 ml of TE. After a 2 ml wash with TE + 0.3 M NaCl the DNA was eluted with two volumes of each 0.5 ml of TE + 0.6 M NaCl. The eluate containing the DNA fragment was diluted with one volume of TE, precipitated with ethanol and dissolved in TEB buffer. The resulting purified protein A gene fragment has cohesive ends corresponding to a Sau 3A restriction site and an intermediate Hind III site.

B4 Preparation of vector plasmid pUR222

Plasmid pUR222 is a commercially avilable vector containing the gene coding for the enzyme β-galactosidase (lac Z). The gene comprises a multilinker having several restriction sites, such as Pst I, Bam H1 and Eco RI. Since β-galactosidase is easily detectable by enzymatic assays, recombinants having a DNA fragment inserted in one of the restriction sites can easily be scored with the use of appropriate host strains. Often Xgal plates are used (Xgal is a chromogenic substrate, 5-bromo-4-chloro-3-indolyl-β-D-galactoside, which releases a blue indolyl derivative when cleaved by β-galactosidase) upon which β-galactosidase negative recombinants appear as white colonies in contrast to the blue-green colour of colonies containing plasmids without an insert.

To cleave plasmid pUR222 in the β-galactosidase coding gene to provide cohesive ends complementary to the cohesive ends of the protein A fragment of step B3 for insertion thereof into the plasmid, the Bam H1 restriction site was used. 1 µg of pUR222, supplied by Boehringer-Mannheim, Germany, was digested with the restriction enzyme Bam H1 for 1 hr at 37°C, whereupon the enzyme was inactivated at 65°C for 10 minutes. This cleavage preparation was used in the

following step B5 for ligation with the protein A fragment.

B5 Construction of a hybrid plasmid pSPA10 containing pSPA2 and pTR262 (Fig. 7)

200 ng of pUR222 digested with Bam H1 as described in step B4 and 200 ng of eluted protein A fragment, as described in step B2, were mixed and ligated in a total volume of 20 µl overnight at +14°C. The enzyme was inactivated at 65°C for 10 minutes, precipitated with ethanol and dissolved in TE buffer. The whole DNA-mixture, containing i.a. recombinant plasmids having the protein A insert in the β-galactosidase gene, was cut with restriction enzymes Hind III and Pst I for 1 hr at 37°C in the recommended buffer for Hind III. This cleaves the recombinant plasmid in the β-galactosidase gene (Pst I) and in the protein A gene (Hind III) producing two fragments, viz. a small fragment consisting of a minor β-galactosidase DNA sequence linked to the part of the protein A gene fragment from the Sau 3A site at position 1.15 kb to the Hind III site in Fig. 2B, and a large fragment consisting of the rest of the recombinant plasmid, which comprises the major part of the β-galactosidase gene linked to the protein A gene fragment from the Hind III site to the Sau 3A site at position 1.8 kb in Fig. 2B. As appears from Fig. 7 the β-galactosidase fragment has an Eco RI restriction site close to the point of fusion with the protein A fragment (the Bam H1 site).

200 ng of plasmid pSPA2 from step B1 were cut with the restriction enzymes Hind III and Pst I in the same way as above to cleave the plasmid into (see Fig. 7) three fragments, viz. one fragment extending from the Hind III site located between the Tet-gene and the 5'-end of the protein A gene to the Hind III site within the protein A gene, a protein A gene fragment extending from the latter Hind III site to the Pst I site at the 3'-end of the protein A gene, and a larger fragment of pTR262 origin comprising the rest of the plasmid.

The two digests prepared above were inactivated at 65°C for 10 minutes, mixed and precipitated with ethanol. The DNA was dissolved in ligation buffer and treated with T4-ligase. The desired recombinant plasmid comprises the above mentioned large fragment, obtained on cleavage of the pUR222 recombinant inserted in pSPA2 between the Hind III site within the protein A gene and the Pst I site and comprising the 5'-end of the protein A gene, one part thereof thus being derived from pSPA2 and the other originating from the pUR222 recombinant. Further, the plasmid is ampicillin and tetracycline resistant and should give blue colour on Xgal plates as will be explained below.

The ligated DNA-mixture was therefore used to transform E. coli RRI del M15. Cleavage, ligation and transformation were effected as described

above. Recombinants were plated out on Xgal plates containing ampicillin and tetracycline. One of the clones appeared as light blue, and restriction analysis was performed on its plasmid. This revealed a plasmid, designated pSPA10 (Fig. 7), which consists of parts of plasmid pUR222, plasmid pTR262 and the protein A gene originating from plasmid pSPA1 and which has a unique Eco RI site at the downstream end of the gene.

Although plasmid pSPA10 does not contain the whole lac Z gene coding for β-galactosidase but only the gene coding for the α-fragment thereof (lac Z'), it is active in cleaving the Xgal substrate thereby producing blue colour under the above used conditions. This is due to a complementation between the α-fragment coded by the plasmid and a chromosomal gene product containing the carboxy terminal fragment of β-galactosidase resulting in an active enzyme. The E. coli RRI del M15 host strain used above has such chromosomal gene material and therefore complements the α-fragment produced by the pSPA10 plasmid to an active β-galactosidase molecule.

B6. Subcloning of the protein A coding gene into plasmid pBR322 for the construction of plasmid pSPA8 (Fig. 7)

1 µg of the purified 2.1 kb protein A fragment from step B2 was cut with restriction enzyme Taq I for 1 hr at 60°C to cleave it within the DNA of staphylococcal origin. The enzyme was inactivated by extraction with an equal volume of phenol, followed by repeated ether extraction and finally the DNA was precipitated with ethanol and dissolved in TE buffer. 1 µg of plasmid pBR322 was cut with restriction enzymes Cla I and Eco RV (which cleave in the same way and thus provide complementary cohesive ends) for 1 hr at 37°C in Bam H1 buffer and then heat inactivated for 10 minutes at 65°C. The DNA samples were mixed, ligated and used to transform E. coli HB101 as described above under Routine Methods. Transformants were streaked out on ampicillin (35 µg/ml). Colonies were picked on plates containing 10 µg/ml of tetracycline and 35 µg/ml of ampicillin, respectively. Transformants that grew on ampicillin but not on tetracycline were considered as recombinants. 4 colonies out of 12 of these recombinants were discovered to be protein A positive using the ELISA method described under Routine Methods. Restriction analysis in which purified plasmid was cut with one, two or three restriction enzymes were performed on one of these clones. The resulting restriction map of this plasmid, designated pSPA8, is shown in Fig. 7. The thus constructed plasmid lacks any E. coli promoter upstream of the protein A gene, the protein A gene fragment being preceded by its own staphylococcal promoter only.

B7 Construction of plasmid pSPA16 (Fig. 8)

A second shuttle vector was constructed coding for a truncated protein A (i.e. lacking the X-region). The construction is schematically outlined in Fig. 8. 5 µg of plasmid pSPA10 from step B5 was cut with Eco RI and HindIII and a 0.4 kb fragment was cut out from a 5% polyacrylamid gel after electrophoresis. The fragment was eluted, and purified as described above under Routine Methods. 5 µg of plasmid pSPA8 from step B6 were treated in the same way and a 0.7 kb fragment was isolated and purified. Finally, 2 µg of plasmid pHV33 were digested with EcoRI, treated with alkaline phosphatase and mixed with the two purified DNA fragments. After treatment with T4-ligase the DNA was used to transform E. coli HB101. Cleavage, alkaline phosphatase treatment, ligation and transformation were performed as described above under Routine Methods. Restriction analysis of 12 ampicillin resistant clones revealed one clone containing plasmid pHV33 with a 1.1 kb insert in the EcoRI site. The plasmid, designated pSPA16, is schematically shown in Fig. 8. Fig. 9 shows the nucleotide sequence and the deduced amino acids preceding the stop codon of this truncated protein A gene. The mature protein lacking region X, thus produced, contains 274 amino acids giving a predicted molecular weight of 30,938. This truncated protein A molecule, which is schematically shown in Fig. 10, contains all the IgG-binding parts of protein A intact except the C-terminal part of region C.

II. Retransformation of shuttle vectors pSPA15 and pSPA16 into E. coli

The shuttle vectors pSPA15 and pSPA16 constructed in section I above were retransformed into E. coli HB101 with selection for ampcillin (amp) resistance (50 µg/ml) as in section I. Transformants were tested for protein A production by the ELISA-test described above under Routine Methods. Plasmid DNA was isolated from protein A positive clones containing the respective plasmids as also described above under Routine Methods.

III. Transformation of strains of S. aureus, S. xylosus and S. epidermidis

A. Preparation and transformation of protoplasts of S. aureus SA113

Different species and even strains of staphylococci contain different restriction and modification systems, and most strains carry several of them (cf. Stobberingh, E.E., and K. Winkler, J. Gen. Microbiol. 99: 359-367 (1977) and Sjöström J.-E. et al., J. Bacteriol. 133: 1144-1149 (1978)).

This causes problems when plasmid DNA isolated from E. coli is to be introduced into staphylococci by transformation.

To overcome the restriction problem a restriction deficient mutant of S. aureus 8325, called SA113, originally isolated by Iordanescu et al (J. Gen. Microbiol. 96: 277-281 (1976)) was therefore used for performing primary

transformations into S. aureus of plasmid DNA isolated from E. coli HB101. The original strain SA113 is lysogenic for prophages Ø11, Ø12 and Ø13 and was furthermore lysogenized with phage 83A. The strain has the following standard phage type: 29/47/75/85/. To further decrease the restriction the protoplasts were heated at 56°C for 30 seconds immediately before the addition of DNA (cf. Asheshov et al. J. Gen. Microbiol. 31: 97-107 (1963), and Sjöström, J.-E., et al., Plasmid 2: 529-535 (1979)).

Methods and media for the preparation of the protoplasts were mainly as those described for Bacillus subtilis by Wyrick and Rogers, J. Bacteriol. 116: 456-465 (1973) as modified by Chang and Cohen, Mol. Gen. Genet. 168: 111-115 (1979). However, some modifications were introduced for staphylococci as described by Lindberg in J. Jeljaczewicz: Staphylococci and Staphylococcal Infections, Zbl. Bakt. Suppl. 10: 535-541; Gustav Fischer Verlag, Stuttgart-New York (1981) and Götz et al, J. Bacteriol. 145: 74-81 (1981).

Ten ml samples of S. aureus SA113 grown in Trypticase Soy Broth (BBL, Cockeysville, Md., USA) to the stationary phase (approx. 2×10^9 colony forming units per ml) were harvested and suspended to the same volume in a hypertonic buffered medium (HBM) consisting of 0.7 M sucrose, 0.02 M Na-maleate and 0.02 M $MgCl_2$, pH 6.5 adjusted with NaOH, plus 43 g Difco Penassay broth powder (Difco Lab., Detroit, Michigan, USA) per liter. Lysostaphin (Schwarz/Mann Orangeburg, N.Y., USA) and lysozyme (Sigma Chemical Co., St. Louis, Mo., USA) were added at 20 and 2000 µg/ml final concentrations, respectively, and the cell suspensions were incubated at 37°C with gentle shaking. Lysozyme is not necessary for removal of the cell wall, but it helps to separate the protoplasts which like intact cells of staphylococci have a tendency to aggregate. This incubation was continued till the absorbancy at 540 nm became constant, which usually occured within 3 hours. The remaining intact bacteria and cell debris were pelletted by centrifugation at 2,500 x g for 10 min. The supernatants were collected and centrifuged again at 16,000 x g for 10 min. The pelletted protoplasts were resuspended in HBM to 1/10 of the volume of the starting culture. 0.4 ml suspensions of the prepared SA113 protoplasts in HBM (approx. 2×10^7 cell wall regenerating protoplasts per ml) were transformed with E. coli plasmid DNA from step II above as follows.

10-20 µg of protein A positive plasmid DNA were added in a maximal volume of 20 µl with gentle mixing. Two milliliters of 40% PEG 6000 (stock solution of polyethylene glycol (PEG) prepared by dissolving 40 g of PEG with a molecular weight of 6,000 (PEG 6000) in 100 ml of hypertonic buffer (HB): 0.7 M sucrose, 0.02 M Na-maleate, and 0.02 M $MgCl_2$, pH 6.5 adjusted with NaOH) was

immediately added followed 2 minutes later by 8 ml of HBM. The suspension was centrifuged at 48,200 x g for 15 min. The pelletted protoplasts were then resuspended in 1 ml of HBM and after appropriate dilutions in HBM samples were plated for regeneration of the cell wall. The regeneration medium was DM3, a Casamino Acids-yeast extract-bovine serum albumin medium containing 0.5 M sodium succinate and 8 g agar per liter according to Chang and Cohen, Mol. Gen. Genet. 168: 111-115 (1979). For selection of chloramphenicol resistant transformants, CY-broth (Novick, R.P., J. Gen. Microbiol. 33: 121-136 (1963)) with 0.5 M sodium succinate, 0.02 M $MgCl_2$, 0.08% bovine serum albumin, and 4 g agar per liter was used as a soft agar overlay with chloramphenicol to give a final concentration of 10 µg/ml in the whole agar medium. Phenotypic expression was allowed at 37°C for 3 hours before the addition of soft agar with chloramphenicol. The plates were incubated at 37°C for 3 days. Chloramphenicol resistant transformants were restreaked on TSA-plates (Trypticase Soy Agar) with chloramphenicol (10 µg/ml).

B. Detection of protein A

A qualitative test of protein A was performed by streaking transformants on Brain-Heart-Infusion (BHI)-agar (Difco lab., Detroit, Michigan, USA) plates with 1% dog serum. Protein A production was detected as a halo of precipitated IgG-protein A complex around the colonies (Kronvall, G. et al., J. Immunol. 104: 140 (1970)). The recipient strain S. aureus SA113 produced very low amounts of protein A, nearly without a detectable halo around the colonies.

C. Preparation of plasmid DNA

Plasmid DNA was prepared from the protein A producing SA113 transformants obtained in step A by a rapid boiling method as described by Holmes et al (Anal. Biochem. 114: 193 (1981)) except that lysozyme was replaced by lysostaphin at a final concentration of 50 µg/ml.

D. Transformation of staphylococci

The following staphylococcal strains were transformed with plasmids pSPA15 and pSPA16 as described above in step A for S. aureus SA113:

Staphylococcus aureus U320, a protein A negative mutant of strain S. aureus SA113 isolated at the Department of Microbiology, The Biomedical Centre, Uppsala, Sweden;

Staphylococcus epidermidis 247, a coagulase negative staphylococcus which does not produce protein A;

Staphylococcus xylosus KL117 a coagulase negative staphylococcus which does not produce protein A.

IV. Production of protein A coded by plasmids pSPA15 and pSPA16

Transformants obtained in steps IIIA and D above were grown in Trypticase Soy Broth enriched with thiamine (1 mg/liter), nicotinic acid (1.5 mg/liter), and Ca-pantothenate (1.5 mg/liter) and the production of extracellular as well as of cell wall bound protein A was determined. Cell wall bound protein A is the amount of protein A released after total lysis of 1 ml of washed cells in the stationary growth phase (approx. 8×10^9 CFU/ml), and extracellular protein A is the amount of protein A in the growth medium.

Cell wall associated protein A was measured quantitatively by testing the binding of ^{125}I-labelled human IgG to the cells (Kronvall, G., J. of Immunol. 104: 273-278 (1970)) or by using the ELISA-test as described under Routine Methods after complete lysis of the cells with lysostaphin.

Extracellular protein A was measured using the ELISA-test.

S. aureus strains Cowan I and A676 were used as reference strains for the production of cell wall bound and extracellular protein A, respectively.

Strain Cowan I has been the type strain for studies of cell wall bound protein A (Nordström K., Acta Universitatis Upsaliensis. Abstracts of Uppsala Dissertations from the Faculty of Medicine 271 (1977)). Small amounts of protein, i.e. extracellular protein A, were found in the growth medium, probably due to autolysis.

Strain A676 produces only extracellular protein A (Lindmark et al, Eur. J. Biochem. 74: 623-628 (1977)) and is used by Pharmacia AB for industrial production of protein A.

The results are presented in Tables 2 and 3 below. All values in the tables are corrected for cell densities and thus directly comparable.

TABLE 2

Production of cell wall bound protein A in different staphylcoccal species coded by plasmid pSPA15

Bacterial strain	Cell wall bound protein A %	Extra-cellular protein A %
Staphylococcus aureus:		
Cowan I	100	12
113	1.5	0
113 (pSPA15)	3	0.3
U320	0	0
U320 (pSPA15)	50	6
Staphylococcus epidermidis:		
247	0	0
247 (pSPA15)	3	0.3
Staphylococcus xylosus:		
KL117	0	0
KL117 (pSPA15)	3	0.3

In Table 2 the amount of cell wall bound protein A in strain Cowan I is set as 100% corresponding to dilution 1/256 in the ELISA-test and equal to 120 mg protein A/liter lysostaphin treated culture. All other figures in the table refer to this figure.

TABLE 3

Production of extracellular protein A in different staphylococcal species coded by plasmid pSPA16

Bacterial strain	Extra-cellular protein A %	Cell wall bound protein A %
Staphylococcus aureus:		
A676	100	0
113	0	1.5
113 (pSPA16)	3	1.5
U320	0	0
U320 (pSPA16)	100	0
Staphylococcus epidermidis:		
247	0	0
247 (pSPA16)	12	0
Staphylococcus xylosus:		
KL117	0	0
KL117 (pSPA16)	25	0

In Table 3 the amount of protein A in the growth medium (i.e. extracellular protein A) is set as 100%, corresponding to dilution 1/256 in the ELISA-test and equal to 90 mg protein A/liter medium. All other figures in the table refer to this figure.

As appears from Tables 2 and 3 above the protein A coded by plasmid pSPA15 is essentially cell wall bound, whereas substantially all the truncated protein A coded by plasmid pSPA16, which lacks region X, is secreted into the growth medium.

Staphylococcus xylosus is used as a starter culture for the production of "Rohwurst" (Liepe, H.-U., Forum Mikrobiologie 5; 10-15 (1982), Fisher et al, Fleischwirtschaft 60: 1046-1051 (1980)) and thus might be considered as an apathogenic staphylococcal species. For this reason S. xylosus containing the cloned protein A gene would be an alternative to S. aureus for industrial production of protein A.

A protein A producing clone of Staphylococcus xylosus KL117 containing the plasmid pSPA16 has been deposited with the collection of the Deutsche

Sammlung von Mikroorganismen (DSM), Grisebachstrasse 8, 3400 Göttingen, Federal Republic of Germany on August 15, 1983 where it was assigned No. DSM 2706.

It is to be understood that the amounts of protein A produced in the above Examples are not maximum yields in any way. Thus, it is within the skill of any person skilled in the art to increase the yields, e.g. by a suitable choice of the nutrient medium etc.

While embodiments of the invention have been presented above the invention is, of course, not restricted thereto, but many variations and modifications of the processes and recombinant matter of this invention are possible without departing from the scope thereof as defined by the subsequent claims.

Thus, the invention is, e.g., also meant to encompass cloning vehicles containing more than one separate deoxynucleotide sequence coding for the desired protein or polypeptide. Further, the invention is intended to comprise also recombinant DNA molecules containing deoxynuclotide sequences, from whatever source obtained, including natural, synthetic or seminsynthetic cources, which are related to the deoxynucleotide sequences coding for protein A or an active fragment thereof, as defined above, by mutation, including single or multiple base substitutions, deletions, insertions and inversions.

International Application No: PCT/ SE83 / 00297

MICROORGANISMS

Optional Sheet in connection with the microorganism referred to on page 17, line 12-16 of the description [1]

A. IDENTIFICATION OF DEPOSIT [2]

Further deposits are identified on an additional sheet ☐ [3]

Name of depositary institution [4]

Deutsche Sammlung von Mikroorganismen

Address of depositary institution (including postal code and country) [4]

Grisebachstrasse 8
D-3400 Göttingen
Federal Republic of Germany

Date of deposit [5]	Accession Number [6]
12 July 1982 (12-07-82)	DSM 2434

B. ADDITIONAL INDICATIONS [7] (leave blank if not applicable). This information is continued on a separate attached sheet ☐

C. DESIGNATED STATES FOR WHICH INDICATIONS ARE MADE [8] (If the indications are not for all designated States)

D. SEPARATE FURNISHING OF INDICATIONS [9] (leave blank if not applicable)

The indications listed below will be submitted to the International Bureau later [9] (Specify the general nature of the indications e.g., "Accession Number of Deposit")

E. ☒ This sheet was received with the International application when filed (to be checked by the receiving Office)

(Authorized Officer) May Rydius

☐ The date of receipt (from the applicant) by the International Bureau [10]

was

(Authorized Officer)

BUREAU OMPI WIPO INTERNATIONAL

WO 84/00773

BUDAPEST TREATY ON THE INTERNATIONAL RECOGNITION OF THE DEPOSIT OF MICROORGANISMS FOR THE PURPOSE OF PATENT PROCEDURE

PCT/SE83/00297

INTERNATIONAL FORM

Martin Lindberg
Department of Microbiology
Biomedical Center
Box 581

S-75123 Uppsala
Schweden

RECEIPT IN THE CASE OF AN ORIGINAL DEPOSIT
issued pursuant to Rule 7.1 by the
INTERNATIONAL DEPOSITARY AUTHORITY
identified at the bottom of this page

I. IDENTIFICATION OF THE MICROORGANISM	
Identification reference given by the DEPOSITOR: E. coli SPA11	Accession number given by the INTERNATIONAL DEPOSITARY AUTHORITY: DSM 2434

II. SCIENTIFIC DESCRIPTION AND/OR TAXONOMIC DESIGNATION

The microorganism identified under I above was accompanied by:

☒ a scientific description

☒ a proposed taxonomic designation

(Mark with a cross where applicable)

III. RECEIPT AND ACCEPTANCE

This International Depositary Authority accepts the microorganism identified under I above, which was received by it on July 12, 1982 (Date of the original deposit)[1]

IV. INTERNATIONAL DEPOSITARY AUTHORITY

Name: DEUTSCHE SAMMLUNG VON MIKROORGANISMEN

Address: Grisebachstrasse 8
D-3400 Göttingen

Signature(s) of person(s) having the power to represent the International Depositary Authority or of authorized official(s):

D. Claus

Date: July 13, 1982

[1] Where Rule 6.4(d) applies, such date is the date on which the status of international depositary authority was acquired; where a deposit made outside the Budapest Treaty after the acquisition of the status of international depositary authority is converted into a deposit under the Budapest Treaty, such date is the date on which the microorganism was received by the international depositary authority.

Form DSM-BP/4 (sole page) 0582

WO 84/00773

BUDAPEST TREATY ON THE INTERNATIONAL RECOGNITION OF THE DEPOSIT OF MICROORGANISMS FOR THE PURPOSE OF PATENT PROCEDURE

PCT/SE83/00297

INTERNATIONAL FORM

Martin Lindberg
Department of Microbiology
Biomedical Center
Box 581

S-75123 Uppsala
Schweden

VIABILITY STATEMENT
issued pursuant to rule 10.2 by the
INTERNATIONAL DEPOSITARY AUTHORITY
identified on the bottom of this page

I. DEPOSITOR	II. IDENTIFICATION OF THE MICROORGANISM
Name: Martin Lindberg Address: Department of Microbiology Biomedical Center Box 581 S-75123 Uppsala, Schweden	Accession number given by the INTERNATIONAL DEPOSITARY AUTHORITY DSM 2434 Date of the deposit or of the transfer: [1] July 12, 1982

III. VIABILITY STATEMENT

The viability of the microorganism identified under II above was tested on [2]
On that date, the said microorganism was

- [X] [3] viable
- [ ] [3] no longer viable

IV. CONDITIONS UNDER WHICH THE VIABILITY TEST HAS BEEN PERFORMED [4]

V. INTERNATIONAL DEPOSITARY AUTHORITY

Name: DEUTSCHE SAMMLUNG VON MIKROORGANISMEN Address: Grisebachstr. 8 D-3400 Göttingen	Signature(s) of person(s) having the power to represent the International Depositary Authority or of authorized official(s): Date: July 13, 1982

[1] Indicate the date of original deposit or, where a new deposit or a transfer has been made, the most recent relevant date (date of the new deposit or date of the transfer).

[2] In the cases referred to in Rule 10.2(a)(ii) and (iii), refer to the most recent viability test.

[3] Mark with a cross the applicable box.

[4] Fill in if the information has been requested and if the results of the tests were negative.

BURE
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INTERNAT

Form DSM-BP 9 (sole page) 0582

CLAIMS

1. A recombinant DNA molecule, characterized by comprising at least one deoxynucleotide sequence coding for protein A or a polypeptide fragment thereof capable of binding at least one immunoglobulin at the Fc-part thereof.

2. The recombinant DNA molecule of claim 1, characterized in that said deoxynucleotide sequence coding for protein A or a polypeptide fragment thereof is derived from a staphylococcal donor.

3. The recombinant DNA molecule of claim 2 characterized in that said staphylococcal donor is a strain of Staphylococcus aureus.

4. The recombinant DNA molecule of any one of claims 1-3, characterized in that said deoxynucleotide sequence comprises a functional signal DNA sequence.

5. The recombinant DNA molecule of claim 4, characterized in that said signal sequence is that of the protein A coding gene of the staphylococcal donor.

6. The recombinant DNA molecule of any one of claims 2-5, characterized in that said deoxynucleotide sequence comprises the promoter sequence of the protein A coding gene of a protein A producing staphylococcal donor.

7. The recombinant DNA molecule of any one of claims 2-6, characterized in that said deoxynucleotide sequence is derived from E. coli SPA11, DSM No. 2434.

8. The recombinant DNA molecule of any one of claims 1-7, characterized in that two or more of said deoxynucleotide sequences together code for an oligomeric protein or polypeptide of protein A and/or active fragments thereof, or a precursor thereto.

9. The recombinant DNA molecule of any one of claims 1-8, characterized in that it is a recombinant plasmid.

10. A process for preparing the recombinant DNA molecule of any one of claims 1-9, characterized by inserting said at least one deoxynucleotide sequence coding for protein A or a polypeptide fragment thereof capable of binding at least one immunoglobulin at the Fc-part thereof into a cloning vehicle.

11. The process of claim 10, characterized in that said deoxynucleotide sequence is obtained by digesting staphylococcal DNA with at least one restriction enzyme.

12. The process of claim 10 or 11, characterized in that it comprises one or more subcloning steps.

13. A microorganism transformed by the recombinant DNA molecule of any one of claims 1-9.

14. The microorganism of claim 13, characterized in that it is a bacterium, preferably a gram-positive bacterium.

15. The microorganism of claim 12, characterized in that it is a Bacillus or Staphylococcus strain.

16. The microorganism of claim 15, characterized in that it is a strain of Bacillus subtilis.

17. The microorganism of claim 15, characterized in that it is a strain of Staphylococcus xylosus.

18. The microorganism of claim 13, characterized in that it is a yeast.

19. A process for preparing the transformed microorganism of any one of claims 13-16, characterized by introducing into a host organism the recombinant DNA molecule of any one of claims 1-9.

20. The process of claim 19, characterized in that it comprises at least one subcloning step.

21. A process for preparing protein A or a derivative thereof capable of binding at least one immunoglobulin at the Fc-portion thereof, characterized by culturing the transformed microorganism of any one of claims 13-18 in a suitable nutrient medium and isolating the desired product formed.

22. Protein A or a derivative thereof capable of binding at least one immunoglobulin at the Fc-portion thereof, characterized by being produced by the process of claim 21.

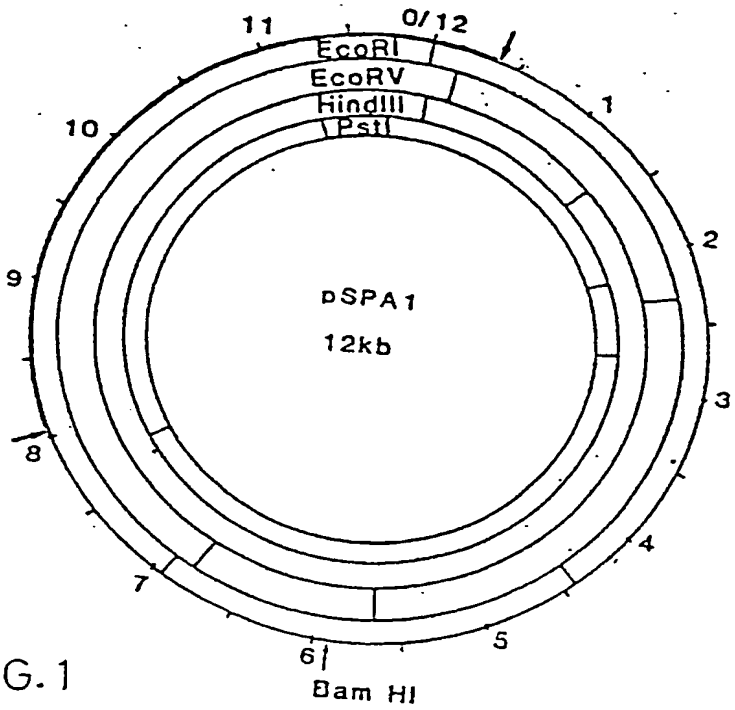
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FIG.1

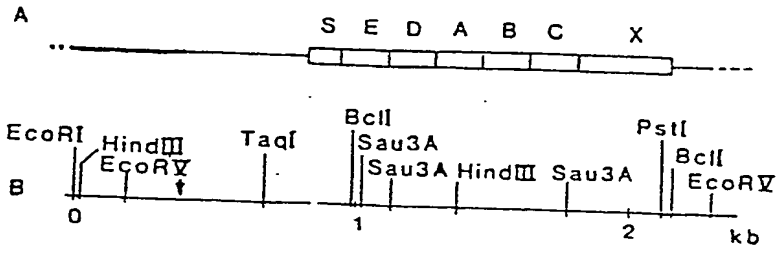
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FIG. 2

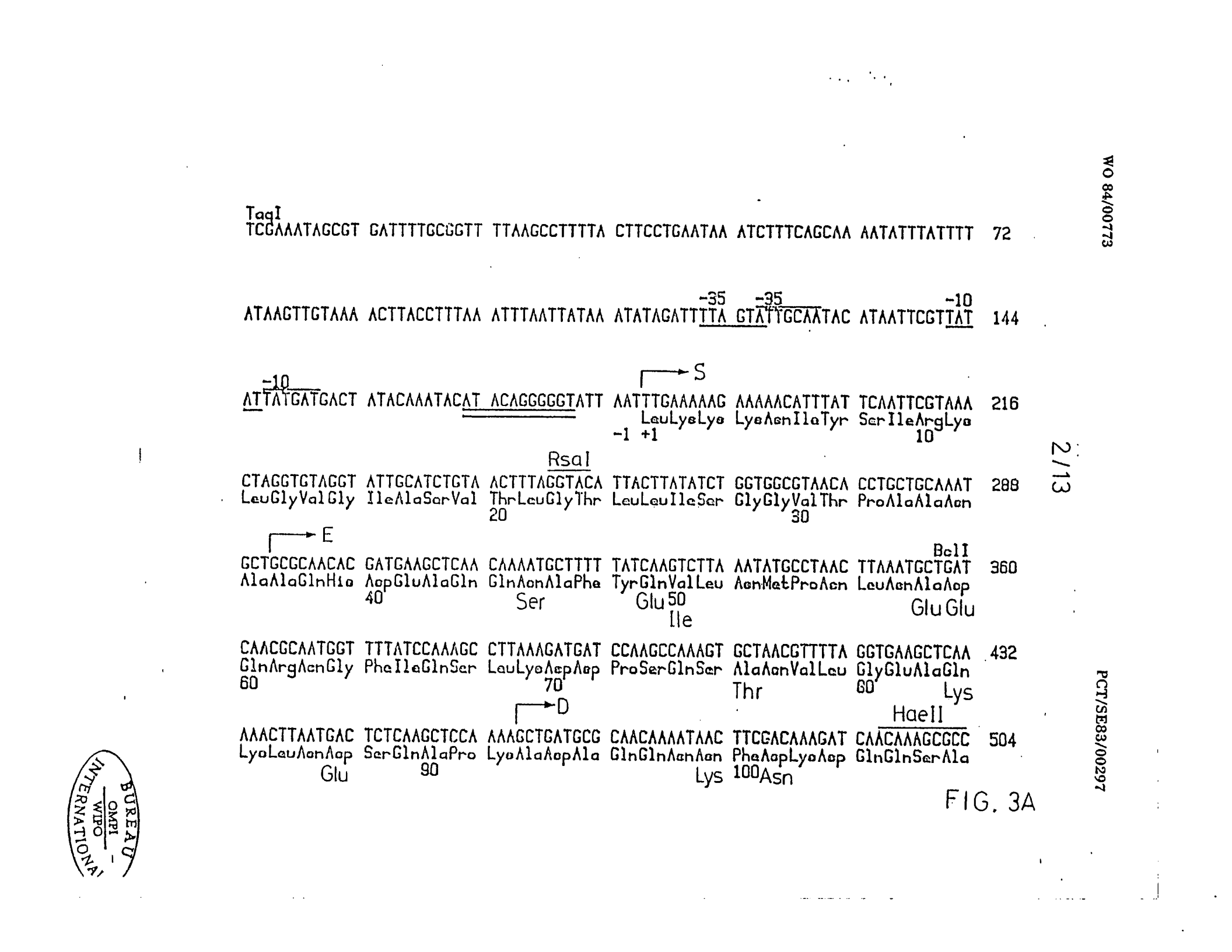

FIG. 3A

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TTCTATGAAATC TTGAACATGCCT AACTTAAACGAA GCGCAACGTAAC GGCTTCATTCAA AGTCTTAAAGAC  576
PheTyrGluIle LeuAsnMetPro AsnLeuAsnGlu AlaGlnArgAsn GlyPheIleGln SerLeuLysAsp
      110                             120                             130
                                      Glu
                                                                  ┌──►A
GACCCAAGCCAA AGCACTAACGTT TTAGGTGAAGCT AAAAAATTAAAC GAATCTCAAGCA CCGAAAGCTGAT  648
AspProSerGln SerThrAsnVal LeuGlyGluAla LysLysLeuAsn GluSerGlnAla ProLysAlaAsp
                         140                             150

AACAATTTCAAC AAAGAACAACAA AATGCTTTCTAT GAAATCTTGAAT ATGCCTAACTTA AACGAAGAACAA  720
AsnAsnPheAsn LysGluGlnGln AsnAlaPheTyr GluIleLeuAsn MetProAsnLeu AsnGluGluGln
             160                             170

                HindIII
CGCAATGGTTTC ATCCAAAGCTTA AAAGATGACCCA AGCCAAAGTGCT AACCTATTGTCA GAAGCTAAAAAG  792
ArgAsnGlyPhe IleGlnSerLeu LysAspAspPro SerGlnSerAla AsnLeuLeuSer GluAlaLysLys
180                             190                             Ala 200

                                ┌──►B
TTAAATGAATCT CAAGCACCGAAA GCGGATAACAAA TTCAACAAAGAA CAACAAAATGCT TTCTATGAAATC  864
LeuAsnGluSer GlnAlaProLys AlaAspAsnLys PheAsnLysGlu GlnGlnAsnAla PheTyrGluIle
                    210                             220
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FIG. 3B

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TTACATTTACCT AACTTAAACGAA GAACAACGCAAT GGTTTCATCCAA AGCCTAAAAGAT GACCCAAGCCAA  936
LeuHisLeuPro AsnLeuAsnGlu GluGlnArgAsn GlyPheIleGln SerLeuLysAsp AspProSerGln
      230                               240                               250

                                                    ┌──►C
AGCGCTAACCTT TTAGCAGAAGCT AAAAAGCTAAAT GATGCTCAAGCA CCAAAAGCTGAC AACAAATTCAAC  1008
SerAlaAsnLeu LeuAlaGluAla LysLysLeuAsn AspAlaGlnAla ProLysAlaAsp AsnLysPheAsn
                          260                               270     Asn

AAAGAACAACAA AATGCTTTCTAT GAAATTTTACAT TTACCTAACTTA ACTGAAGAACAA CGTAACGGCTTC  1080
LysGluGlnGln AsnAlaPheTyr GluIleLeuHis LeuProAsnLeu ThrGluGluGln ArgAsnGlyPhe
                280                               290

ATCCAAAGCCTT AAAGACGATCCT TCGGTGAGCAAA GAAATTTTAGCA GAAGCTAAAAAG CTAAACGATGCT  1152
IleGlnSerLeu LysAspAspPro SerValSerLys GluIleLeuAla GluAlaLysLys LeuAsnAspAla
300                               310                               320

                ┌──►X
CAAGCACCAAAA GAGGAAGACAAT AACAAGCCTGGC AAAGAAGACAAT AACAAGCCTGGC AAAGAAGACAAT  1224
GlnAlaProLys GluGluAspAsn AsnLysProGly LysGluAspAsn AsnLysProGly LysGluAspAsn
                      330                               340

AACAAGCCTGGC AAAGAAGACAAC AACAAGCCTGGC AAAGAAGACAAC AACAAGCCTGGT AAAGAAGACAAC  1296
AsnLysProGly LysGluAspAsn AsnLysProGly LysGluAspAsn AsnLysProGly LysGluAspAsn
      350                               360                               370

AACAAGCCTGGC AAAGAAGACGGC AACAAGCCTGGT AAAGAAGACAAC AAAAAACCTGGT AAAGAAGATGGC  1368
AsnLysProGly LysGluAspGly AsnLysProGly LysGluAspAsn LysLysProGly LysGluAspGly
                          380                               390
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FIG. 3C

```
AACAAGCCTGGT AAAGAAGACAAC AAAAAACCTGGT AAAGAAGACGGC AACAAGCCTGGC AAAGAAGATGGC 1440
AsnLysProGly LysGluAspAsn LysLysProGly LysGluAspGly AsnLysProGly LysGluAspGly
             400                       410

AACAAACCTGGT AAAGAAGATGGT AACGGAGTACAT GTCGTTAAACTT GGTGATACAGTA AATGACATTGCA 1512
AsnLysProGly LysGluAspGly AsnGlyValHis ValValLysLeu GlyAspThrVal AsnAspIleAla
420                       430                                     440

                                    PstI                                BclI
AAAGCAAACGGC ACTACTGCTGAC AAAATTGCTGCA GATAACAAATTG GCTGATAAAAAC ATGATCAAACTT 1584
LysAlaAsnGly ThrThrAlaAsp LysIleAlaAla AspAsnLysLeu AlaAspLysAsn MetIleLysLeu
             450                       460

                                    ---------       ------ -----
GGTCAAGAACTT GTTGTTGATAAG AAGCAACCACAA ACCATGCAGAGC TAACAAAGCTCA AGCATTATCAGA 1656
GlyGlnGluLeu ValValAspLys LysGlnProGln ThrMetGlnSer ***
     470                               480

AACTGGCGAAGA AAATCCATTCAT CGGTACAACTGT ATTTGGTGGATT ATCATTAGCCTT AGGTGCAGCGTT 1728

                                                    EcoRV
ATTAGCTGGACG TCGTCGCGAACT ATAAAAACAAAC AATACACAACGA TAGATATC                   1784
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FIG. 3D

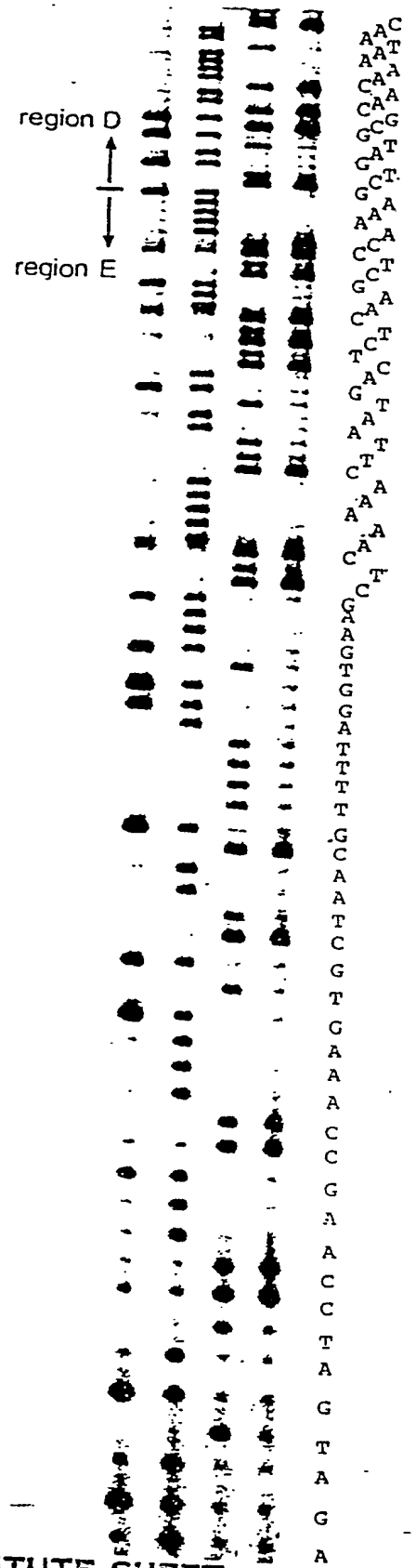

FIG. 4

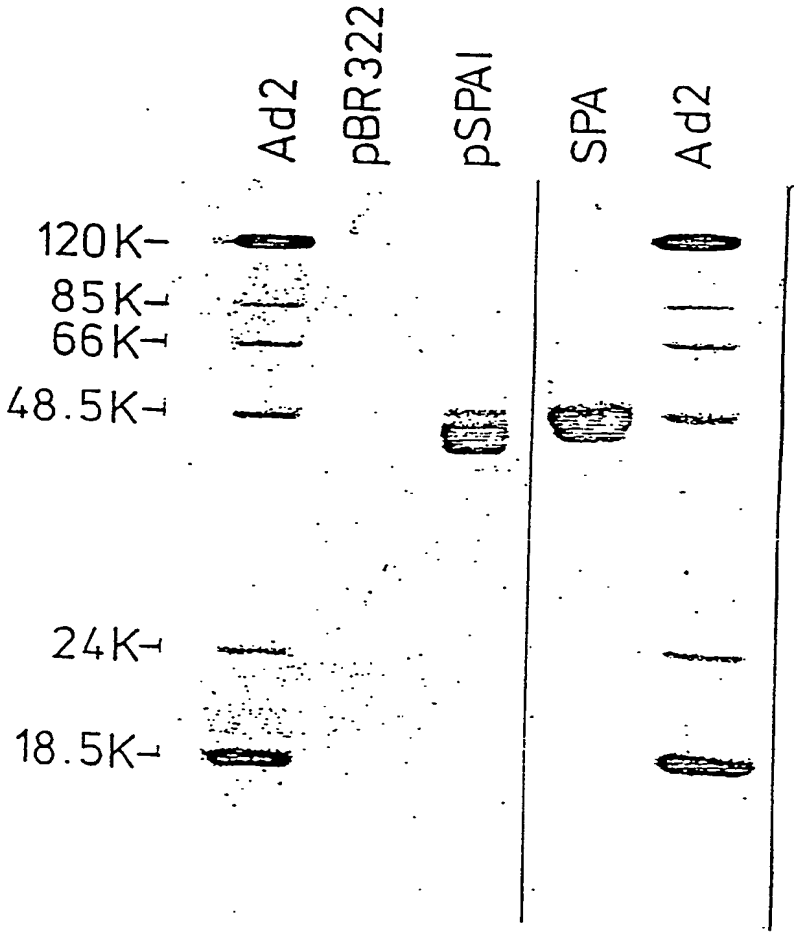

FIG. 5

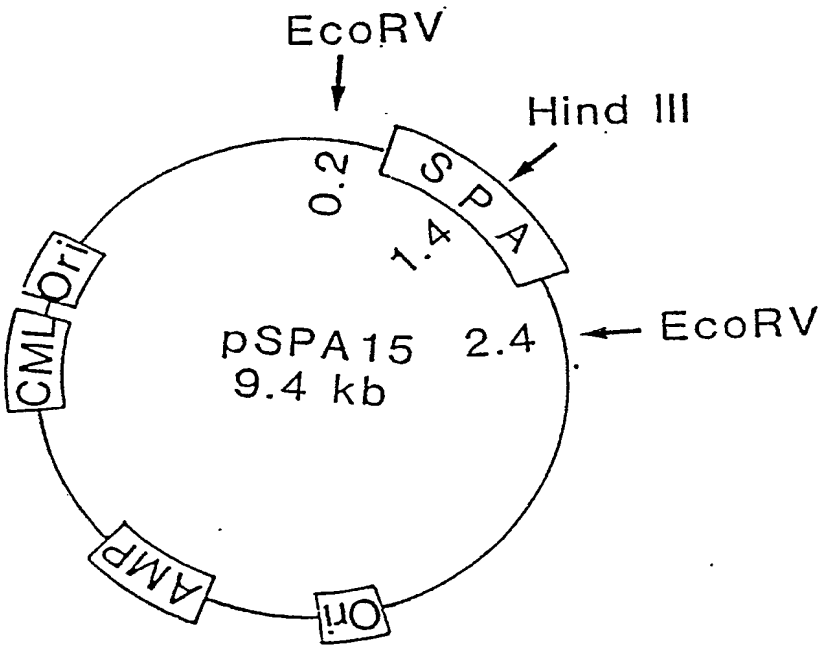

FIG. 6

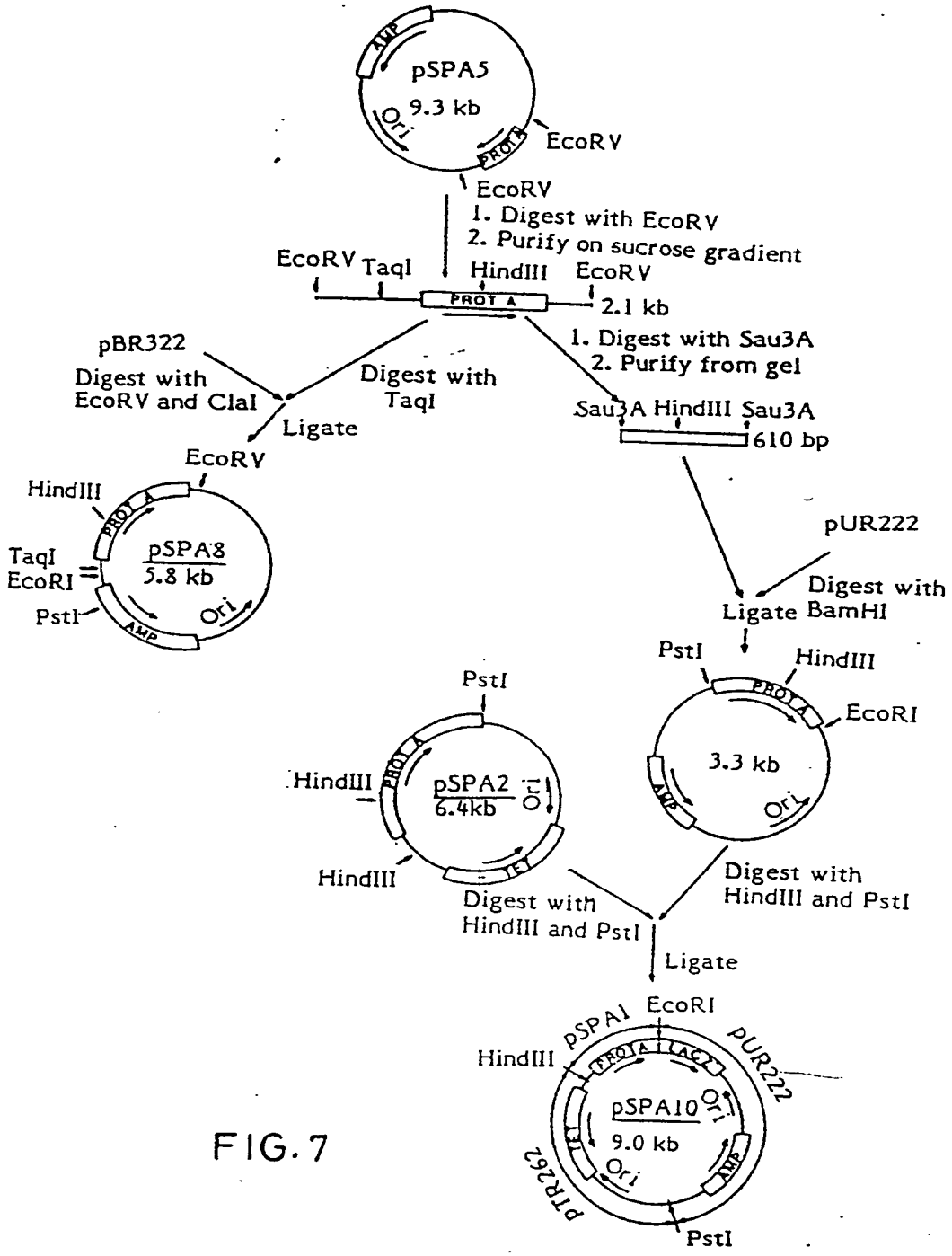
pSPA5
9.3 kb
AMP
Ori
PROT A
EcoRV
EcoRV
1. Digest with EcoRV
2. Purify on sucrose gradient
EcoRV TaqI
HindIII EcoRV
PROT A
2.1 kb
pBR322
Digest with
EcoRV and ClaI
Digest with
TaqI
Ligate
1. Digest with Sau3A
2. Purify from gel
Sau3A HindIII Sau3A
610 bp
EcoRV
HindIII
PROT A
TaqI
EcoRI
pSPA8
5.8 kb
PstI
AMP
Ori
pUR222
Digest with
Ligate BamHI
PstI
HindIII
PROT A
EcoRI
3.3 kb
AMP
Ori
PstI
HindIII
PROT A
pSPA2
6.4kb
Ori
HindIII
Digest with
HindIII and PstI
Digest with
HindIII and PstI
Ligate
EcoRI
pSPA1
HindIII
PROT A
LAC
pUR222
pSPA10
9.0 kb
Ori
TET
Ori
AMP
pTR262
PstI

FIG. 7

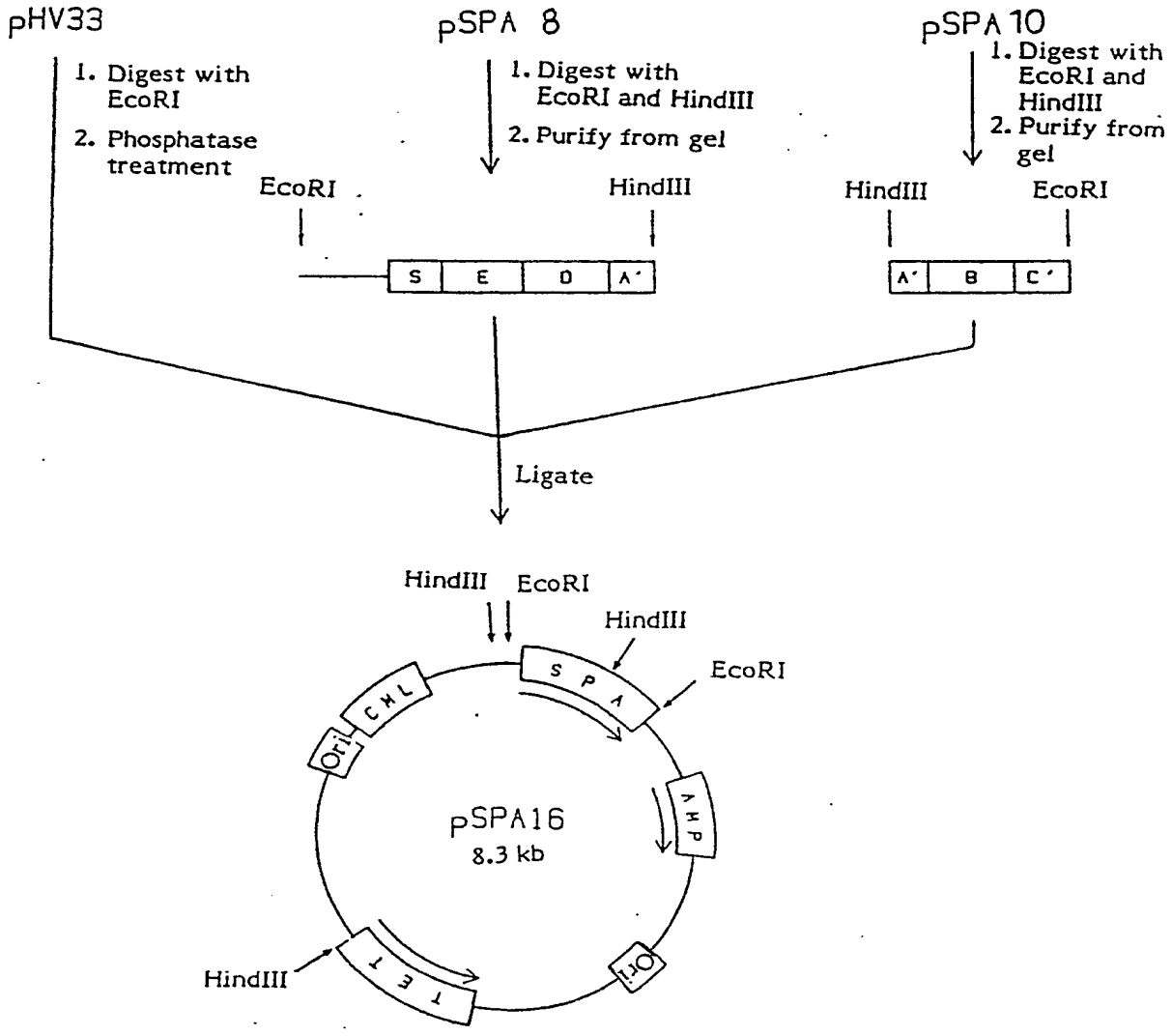

FIG. 8

SUBSTITUTE SHEET

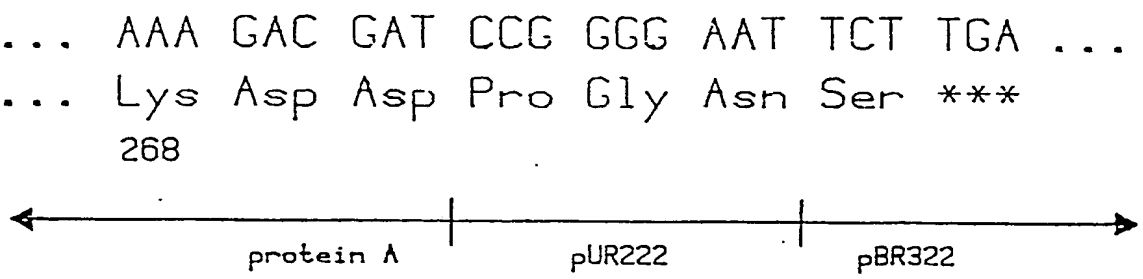

FIG. 9

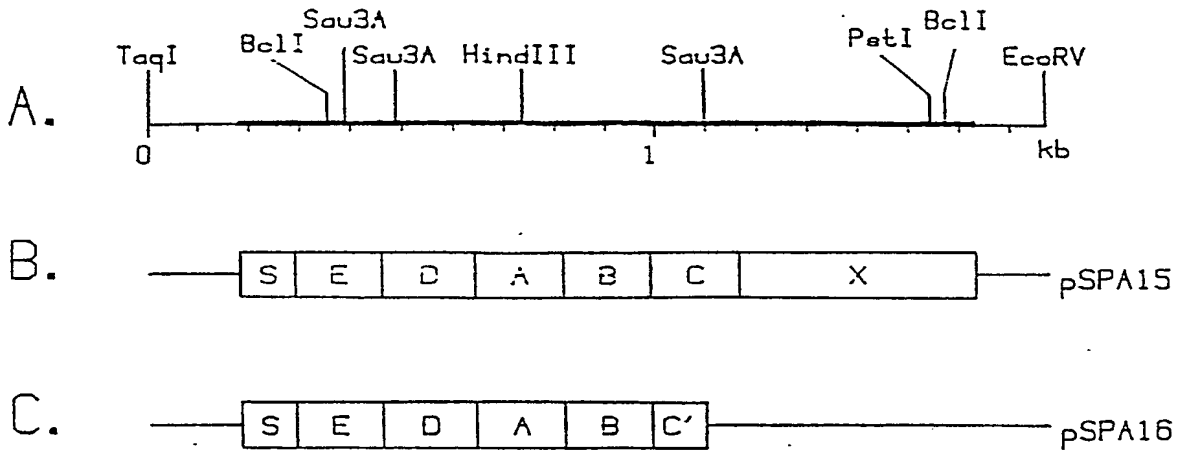

FIG. 10

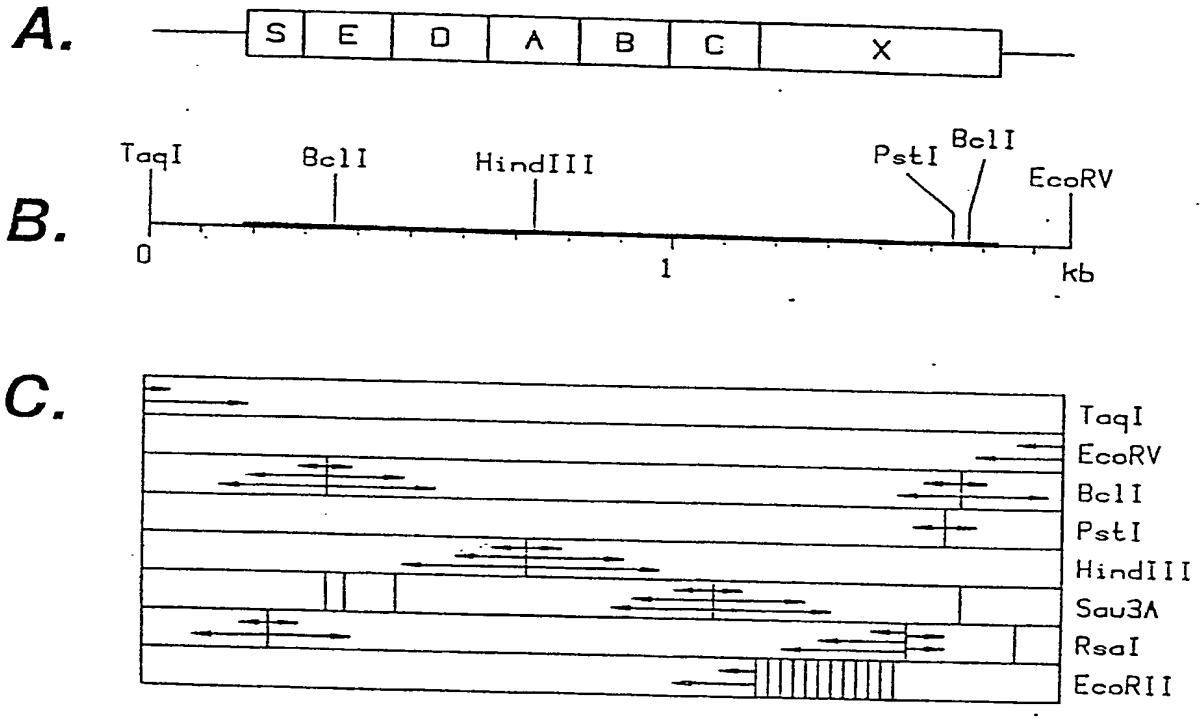

FIG. 11

INTERNATIONAL SEARCH REPORT

International Application No PCT/SE83/00297

I. CLASSIFICATION OF SUBJECT MATTER (If several classification symbols apply, indicate all) [3]

According to International Patent Classification (IPC) or to both National Classification and IPC 3

C 12 N 15/00, C 12 P 21/02

II. FIELDS SEARCHED

Minimum Documentation Searched [4]

Classification System	Classification Symbols
IPC 3	C 12 N 15/00, C 12 P 21/00,02
US Cl	435: 172

Documentation Searched other than Minimum Documentation to the Extent that such Documents are included in the Fields Searched [5]

SE, NO, DK, FI classes as above

III. DOCUMENTS CONSIDERED TO BE RELEVANT [14]

Category [6]	Citation of Document, [16] with indication, where appropriate, of the relevant passages [17]	Relevant to Claim No. [18]
P	Proc. Natl. Acad. Sci USA Vol 80, pp 697-701, published February 1983 (LÖFDAHLS S ET AL GENE FOR STAPHYLOCOCCAL PROTEIN A)	1-22
A	Chemical Abstacts Vol 87 (1977), abstract No 150071j, Eur. J. Biochem 1977, 78 (2), 471-90	1
A	Chemical Abstracts Vol 86 (1977), abstract No 135207u, Eur. J. Biochem 1977, 73(2), 343-51	

[6] Special categories of cited documents: [15]

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

IV. CERTIFICATION

Date of the Actual Completion of the International Search [8]	Date of Mailing of this International Search Report [9]
1983-11-21	1983 -11- 24
International Searching Authority [1]	**Signature of Authorized Officer [10]**
Swedish Patent Office	Carl Olof Gustafsson